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**An Investigation into Placental Protein 14, a Modulator of the Immune  
Response associated with Human Reproduction**

**By**

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**A thesis submitted to Sheffield Hallam University in partial fulfilment  
of the requirements for the degree of Doctor of Philosophy**

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**September 1994**



## ABSTRACT

This thesis describes investigations into Placental Protein 14 (PP14), a immunomodulator involved in human reproduction. The studies included the development of a purification procedure and an investigation of the activity of the protein. In addition the cDNA coding for the protein was cloned and expressed as a recombinant fusion protein and the molecular structure of the protein was predicted and analysed using computer-assisted modelling. Finally the clinical significance of the protein was studied in a range of patient groups.

The purification scheme consisted of ion exchange, hydrophobic interaction and gel filtration chromatography, and the pure protein obtained was analysed by SDS-PAGE and Western blotting. The results demonstrate that the purification procedure is a suitable method to obtain PP14 in large quantity and with high purity. PP14 purified by this method retained its activity and was shown to suppress, in a dose-dependent manner, the uptake of  $^3\text{H}$ -Thymidine by peripheral blood mononuclear cells stimulated with interleukin-2. Purified PP14 was also shown to suppress the uptake of  $^3\text{H}$ -Thymidine by the cell line U937, also in a dose-dependent manner. This suppression could be removed by the incubation of the PP14 sample with an immunoabsorbent gel linked to monoclonal antibodies against PP14, demonstrating that PP14 was the molecule responsible for the observed activity. Based on the suppression by PP14 of U937 cell growth a bioassay for PP14 was developed, this assay was used to express the specific activity of PP14 in Units/ml.

To obtain recombinant PP14, mRNA was purified from a tissue sample and reverse transcription used to prepare cDNA. Specific primers were used to amplify the portion of cDNA coding for PP14 which was then ligated into the plasmids pUC 18 and pGEX-KG. Recombinant PP14 was then expressed as a fusion protein with glutathione-S-transferase. The expression conditions were optimised and the fusion protein was purified using affinity chromatography.

The structure of PP14 was investigated using computer assisted modelling. PP14 is a member of the lipocalin family of proteins which share the feature of binding small hydrophobic molecules. The X-ray coordinates of two lipocalins known to share sequence homology with PP14 were used as a basis to model a predicted structure for PP14. An analysis of the structural motifs of the protein was carried out, and it was established that PP14 shares many of the characteristic features of this family of proteins including the presence of a binding pocket. The model was then used to predict potential ligands for PP14.

PP14 was measured by radioimmunoassay in uterine flushings from fertile women, women with unexplained infertility and women suffering from recurrent miscarriages, and in plasma samples from fertile and infertile women. The results from the uterine flushings from fertile women showed that PP14 levels rose during the second half of the menstrual cycle reaching  $\mu\text{g/ml}$  levels by the end of the cycle. These physiological concentrations are in the same range as the concentrations at which the immunomodulatory activity of PP14 was observed *in vitro*. The levels of PP14 measured in uterine flushings were lower in infertile women than in fertile women, indicating that a deficiency in PP14 may be associated with infertility. The levels measured in plasma samples from these two groups of women did not pick up this difference. These results suggests that the measurement of proteins such as PP14 in uterine flushings instead of plasma samples may be a more sensitive indicator of local uterine function. In women suffering from recurrent miscarriage a significant lack of secretion of PP14 was observed around the time of implantation. This may be connected with the failure of implantation in these patients. A correlation was observed between the PP14 levels measured in uterine flushings from recurrent miscarriage patients and the level of endometrial development.

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Li TC, Dalton C, Bolton AE, Ling E, Warren A & Cooke ID (1992). An analysis of the variation of plasma concentrations of placental protein 14 in artificial cycles.

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# LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
Ag	antigen
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
βLG	beta-lactoglobulin
bp	base pairs
BSA	bovine serum albumin
°C	degrees Celsius
CaCl <sub>2</sub>	calcium chloride
CD	cluster of differentiation
cDNA	complementary DNA
Ci	Curie
cm	centimetre
CNBr	cyanogen bromide
CO <sub>2</sub>	carbon dioxide
cpm	counts per minute
CSF	colony stimulating factor
CTP	cytidine triphosphate
CV	coefficient of variation
Da	daltons
DEAE	diethylaminoethyl
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
FPLC	Fast Protein Liquid Chromatography
FSH	follicle stimulating hormone
g	gram
Glu	glutamic acid
Gly	glycine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione <i>S</i> -transferase
GTP	guanosine triphosphate
<sup>3</sup> H	tritium
hCG	human chorionic gonadotrophin
HCl	hydrochloric acid
HLA	human leukocyte antigen
IL-1	interleukin 1
IL-2	interleukin 2
IL-3	interleukin 3
IL-6	interleukin 6

$\gamma$ -IFN	$\gamma$ -interferon
IPTG	$\beta$ -D-isopropylthiogalactopyranoside
IVF	<i>in vitro</i> fertilisation
KCl	potassium chloride
kb	kilobase
kDa	kilodalton
l	litre
LA	Luria agar
LAK	lymphokine activated killer
LB	Luria broth
LGL	large granular lymphocyte
LH	luteinising hormone
LIF	leukaemia inhibitory factor
M	molar
mA	milliamp
mg	milligram
MgCl <sub>2</sub>	magnesium chloride
MHC	major histocompatibility complex
ml	millilitre
MLR	mixed lymphocyte reaction
mM	millimolar
mRNA	messenger RNA
MUP	major urinary protein
MW	molecular weight
NaCl	sodium chloride
NaI	sodium iodide
NaOH	sodium hydroxide
NaN <sub>3</sub>	sodium azide
Na <sub>2</sub> SO <sub>3</sub>	sodium sulphate
NBT	nitro blue tetrazolium
ng	nanogram
NK	natural killer
nm	nanometre
<i>P</i>	probability
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE	prostaglandin E
pI	isoelectric point
PMNL	polymorphonuclear leukocyte
pmol	picomole
PMSF	phenylmethylsulphonyl fluoride
PP14	Placental protein 14
RBP	retinol-binding protein
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
S.E.M.	standard error of the mean
SDS	sodium dodecyl sulphate
TAE	Tris-Acetate-EDTA buffer

TBE	Tris-Borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	transforming growth factor
Thr	threonine
TLX	trophoblast-lymphocyte cross reactive
TNF	tumour necrosis factor
Tris	trishydroxymethylaminomethane
Trp	tryptophan
TTP	thymidine triphosphate
Tyr	tyrosine
U	Units
μCi	microcurie
μg	microgram
μl	microlitre
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside
ZP3	zona pellucida protein 3
< less than	
> more than	

# CHAPTER ONE: INTRODUCTION

Pregnancy is a unique physiological situation in which a partly foreign tissue survives and develops within the maternal body. During the process of implantation the fetal trophoblast containing paternal genes invades deep into the maternal decidual tissue, which is immunologically active and contains many leukocytic cells, some of which appear to be specific populations found only during pregnancy. Despite the expression by some trophoblast cells of paternal antigens, there is no evidence for rejection of the fetus by the maternal immune system in normal pregnancy. The specific leukocytic cell populations may play a role in limiting the invasion of the trophoblast and in the protection against maternal rejection. Decidual tissue also contains immunosuppressive molecules which may also contribute to the local down-regulation of the maternal immune system. A situation in which either the leukocytic cells are over-activated or the protective immunosuppressive environment is compromised may lead to immunological imbalance causing trophoblast lysis and miscarriage (*Hill, 1990*).

One of the candidate molecules for a role in the modulation of the local immunological activity in the decidua during pregnancy is placental protein 14 (PP14). This molecule is present in substantial quantities in the uterine environment at the time of implantation and during the first trimester of pregnancy (*Julkunen et al., 1985*) and has been shown to have immunosuppressive activity (*Bolton et al., 1987*).

Chapter 1 of this thesis reviews the immunological interactions that occur in the endometrium and decidua leading up to and during the first stages of pregnancy. This background information gives a context for the role of PP14, which may be involved in the regulation of these interactions.

The work described in Chapters 2 and 3 relates to the purification and biological activity of PP14. Chapters 4 and 5 describe recombinant expression and studies of the molecular

structure of PP14. Chapter 6 describes the measurement of PP14 in fertile women, patients with unexplained infertility and patients with recurrent miscarriage.

## **1.1 IMPLANTATION AND THE ESTABLISHMENT OF PREGNANCY**

Implantation depends on the successful synchronisation and completion of a number of processes including the development and release of the ovum and the preparation of the endometrium to provide an optimum environment for the dividing blastocyst. After fertilisation the presence of the embryo is signalled to the endometrium. The embryo must then undergo adhesion, attachment and penetration to establish a blood supply to access nutrients from the mother.

### **1.1.1 The menstrual cycle**

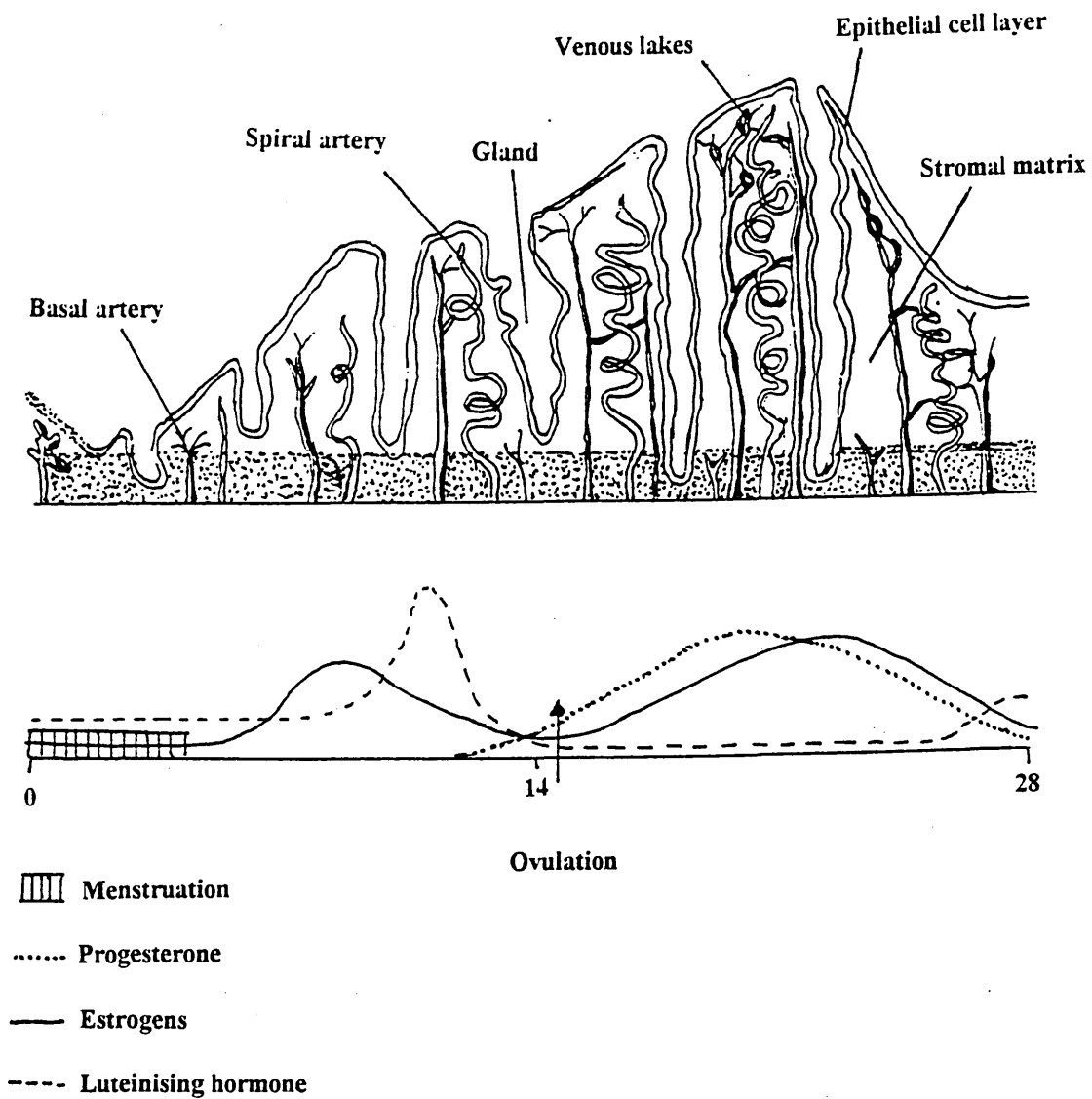
In humans the endometrium undergoes a constant cyclical process of breakdown regeneration and growth during the menstrual cycle. This cycle lasts between 26 and 30 days in most women and is under hormonal control. The cycle starts with menstruation and the first day of bleeding is designated day 1 of the cycle. For the next 14 days until ovulation the cycle is under the influence of oestrogen, during the second half of the cycle until day 28, progesterone is the dominant hormone.

### **1.1.2 The Endometrium**

The uterus consists of a thick myometrium of smooth muscle arranged in layers. Lining the muscle is the endometrium consisting of a stromal matrix over which lies a layer of epithelial cells facing the uterine cavity. Glands lined with epithelial cells penetrate the stromal cell layer. Within the endometrium are spiral arteries which provide it with a rich blood supply and venous lakes (Figure 1.1).

The first 14 days of the menstrual cycle are a phase of endometrial cellular proliferation. At the beginning of the menstrual cycle, proteases break down the stroma and the endometrium is shed leaving a denuded surface with only the cups forming the bottom of the glands left. The blood vessels are also lost in this process. Within 5 days the endometrium is re-epithelialised, and by day 21 the stroma and glands have regrown and





**Figure 1.1:** The changes in human endometrium during the menstrual cycle. The underlying hormone changes are also shown. Redrawn from Johnson & Everitt (1988).

the tissue surface is covered with microvilli which provide a receptive environment for implantation (*Nilsson et al., 1980*).

The second 14 days of the cycle are a secretory phase during which proliferation slows down and the epithelial cells secrete a watery fluid, containing proteins and nutrients for the growing embryo, into the uterine lumen (*Nilsson et al., 1980*). The function of many of these molecules is unknown but some may be involved in the regulation of endometrial or trophoblastic growth.

After day 14 if fertilisation has occurred, the stromal compartment of the endometrium undergoes the process of decidualisation as the cells enlarge and become glycogen and lipid rich (*Noyes et al., 1950; Wewer et al., 1985*). The surface microvilli of the epithelial cell layer become flattened and bulbous cytoplasmic projections appear (*Lindenberg, 1991*). The tissue is known as the decidua from this point on. If fertilisation has not occurred the endometrium still undergoes these changes but to a lesser degree in a process known as pre-decidualisation.

In a conception cycle the pre-embryo passes through the fallopian tube into the uterus and implants into the decidualised endometrium which will continue to develop. This development is dependent on progesterone, secreted from the corpus luteum in response to chorionic gonadotrophin released from the embryo. In a non-conception cycle the unfertilised egg is passed out of the reproductive tract and the endometrium is shed at day 28 as a consequence of the drop in progesterone levels.

### **1.1.3 Ovulation**

While the endometrium develops during the proliferative phase of the cycle, the ovary undergoes a phase of follicular development. Oocytes are formed before birth, and undergo mitotic proliferation then meiotic division. Shortly after birth the follicles arrest in the first meiotic prophase as primordial follicles (*Peters et al., 1975*). These follicles

remain in arrested meiotic prophase awaiting the signal to resume development until puberty, when they recommence growth at the rate of a few a day.

When development restarts, the oocytes increase in diameter and the granulosa and thecal cells that surround them divide and become several layers thick. The granulosa cells secrete glycoproteins that form a layer, the zona pellucida, between themselves and the oocyte. Gap junctions formed by cytoplasmic processes that penetrate the zona maintain contact between the granulosa cells and the oocyte. These developing follicles are now termed preantral follicles and their fate depends entirely on the hormonal environment in which they find themselves (*Johnson & Everitt, 1988*). For continued development there must be adequate levels of luteinising hormone (LH) and follicle stimulating hormone (FSH) present in the circulation to bind to receptors on the follicles and induce conversion to antral follicles. If these hormones are not available the oocytes will become atretic.

At the end of the previous menstrual cycle the fall in oestrogen and progesterone allows the secretion of LH and FSH to rise, providing an environment in the beginning of the cycle permissive for the development of antral follicles. This stage of development is characterised by the continued proliferation of the granulosa cells and outer thecal cells. Fluid appears between the granulosa cells and collects around the oocyte forming follicular fluid. A week before ovulation the leading follicle begins to dominate, exhibiting a higher mitotic rate and a rapid expansion of the volume of follicular fluid (*Adashi, 1994*). At this point the follicle is again dependent on the environment for its continuing development. Development will only continue if a surge of LH coincides with the appearance of LH receptors on the outer granulosa cells. The LH surge causes terminal growth changes that result in ovulation and the expulsion of the oocyte from the follicle.

LH also continues to affect the post-ovulation follicle which becomes a corpus luteum producing progesterone. The corpus luteum declines 9-11 days after ovulation unless

chorionic gonadotrophin (hCG) released from the trophoblast intervenes (*Hearn, 1986*). This hormone is released into the bloodstream and travels to the ovary where it binds to the luteinising hormone receptors in the corpus luteum and promotes the continued progesterone production by luteal cells which in turn supports the endometrium and prevents its breakdown.

#### **1.1.4 Implantation**

Fertilisation occurs in the fallopian tube and the conceptus remains at this site for a few days before being passed down the tube to the uterus by the action of the cilia. During this time the morphology of the conceptus changes as it compacts to form a morula. At the 32-64 cell stage, the morula undergoes a rapid series of mitoses during its transition to a blastocyst. The blastocyst consists of an outer rim of trophectoderm cells surrounding a blastocoelic cavity with an inner cell mass placed against one side of the cavity. Transcriptional activity increases as growth factors become active in the embryo (*Edwards, 1994*).

The trophectoderm gives rise to the trophoblast which is the tissue concerned with the interaction with the mother and the nutrition and support of the foetus. Before implantation, while the embryo is still lying free in the uterine luminal fluid, interactions are necessary between the conceptus and the maternal tissue (*Lindenberg, 1991*), involving extracellular signals which pass both ways (*Heald, 1976; Hearn, 1986; Heap et al., 1979*). In humans the embryo begins to implant around day 6 after fertilisation. It has been shown in mice that there is a period of optimal endometrial sensitivity when conditions for implantation are most favourable (*Finn, 1982*). This window of opportunity is dependent on the decidualisation of the endometrium, which is stimulated by the presence of the embryo before physical contact occurs (*Lundkvist & Nilsson, 1984*). One of the molecules involved in this interaction may be leukaemia inhibitory factor (LIF) as the expression of this molecule in the endometrium of mice on the fourth day of pregnancy, is essential for implantation which occurs a few hours later (*Stewart et al., 1992*).

Implantation can be split into two phases (*Lindenberg, 1991*). First there is the positioning and adhesion of the trophoblast cells to the uterine surface. The polarity of the embryo has already developed by the stage at which it attaches to the endometrium (*Lindenberg et al., 1986; 1989*), and the embryo orientates itself so that the site of attachment is on the side of the inner cell mass. The polar trophoctoderm cells involved with primary adhesion to the endometrial cell express specific cell surface adhesion molecules to facilitate this process (*Edwards, 1994*). There are also changes in the carbohydrates on the luminal uterine surface (*Chavez & Anderson, 1985; Anderson et al., 1986*) which may enable the endometrium to recognise and bind the adhesion molecules expressed by the trophoblast.

The second phase is the penetration of the uterine epithelium. There are three main mechanisms of penetration which vary between species: 1. fusion, found in rabbits where the trophoblast fuses with the epithelial cells, 2. intrusive, found in ferrets, where the trophoblast cells extend cytoplasmic protrusions between epithelial cells and 3. displacement, found in humans, rats and mice, where the epithelial cells are lifted off from the stroma and pile up on either side of the implantation site. Because of these species differences, comparisons between implantation in humans and that in other species can only be made with care (*Ramsey et al., 1976*) All these types of implantation have specialised cell-cell contact between trophoblast and surface epithelium of the uterus.

### **1.1.5 Trophoblast invasion**

In humans the trophoblastic tissue invades the wall of the uterus within 24 hours of adhesion first occurring (*Lindenberg et al., 1986; 1989*). The trophoctoderm penetrates the basement membrane underlying the uterine epithelium by breaking down the extra-cellular matrix of the maternal tissues with metalloproteases and collagenases to reach the maternal blood supply. The cells of the trophoctoderm proliferate rapidly as the conceptus migrates through the endometrium into the spiral arteries, replacing the maternal endothelial and smooth muscle cells in the vessel walls (*Brosens et al., 1967*;

*Pijnenborg et al., 1980; Robertson et al., 1986*). However, the invading trophoblast cells do not appear to have cytolytic activity since degenerating epithelial cells have not been observed next to the trophoblast cells (*Lindenberg, 1991*).

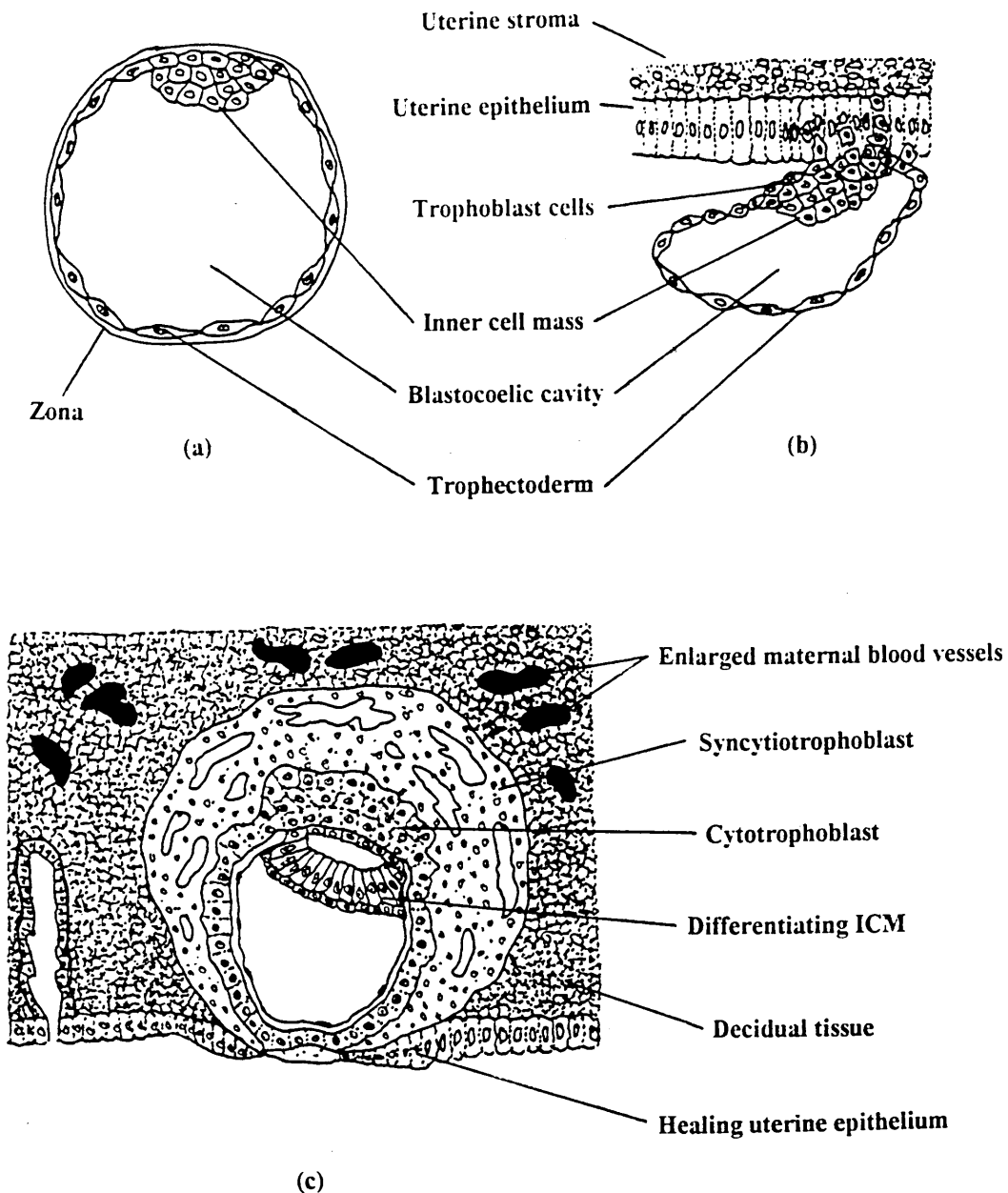
During implantation, the trophoblast cells differentiate into a number of sub-types (Figure 1.2). Some of the outer layer of trophoblast cells fuse to form a multi-nucleated syncytium, the syncytiotrophoblast, across which blood gases and nutrients diffuse to the foetus and waste products to the maternal circulation. Other trophoblast cells remain as single cells and form the cytotrophoblast.

The surface of the fetal tissue is made up of branched villi which anchor it in the maternal decidua. At the tips of the villi there is a break in the syncytiotrophoblast layer and cytotrophoblast cells proliferate from these villi into the maternal spiral arteries eroding the vessel walls so that later in pregnancy they will be able to expand and accommodate the increased maternal blood flow to the foetus.

The invasion of maternal tissue occurs in two stages, one at this first encounter between the embryo and mother in the first trimester, and a second stage in the second trimester, when the cytotrophoblast cells invade the myometrium underlying the decidual tissue. The failure of the first wave of invasion can lead to first trimester miscarriage (*Khong et al., 1987*), and failure of the second to inter-uterine growth retardation or pre-eclampsia (*Robertson et al., 1986; Khong et al., 1986*).

In addition to the invasion of the spiral arteries cytotrophoblast cells break off from the villi and migrate deep into the maternal tissue becoming extravillous trophoblast cells. The reason for this is unclear, but these cells may be involved in signalling the presence of the foetus to the maternal immune system.

The only fetal tissues in direct contact with the maternal blood and tissues are the syncytiotrophoblast, cytotrophoblast and extravillous trophoblast cells.



**Figure 1.2:** Schematic representation of implantation.

(a) 4-5 days after fertilisation, free-living blastocyst; (b) 6 days, trophectoderm transforms to trophoblastic tissue and attaches to epithelial cells then starts to penetrate epithelium; (c) 9 days, decidualisation in underlying stromal tissue spreads out from the attachment site, trophoblast erodes epithelium and invades decidual tissue becoming embedded. Redrawn from Johnson & Everitt, (1988)

Syncytiotrophoblast is bathed by the maternal blood supply, and the extravillous cytotrophoblast cells are in contact with the maternal decidua.



## **1.2 IMMUNOLOGY OF THE MATERNO-FETAL RELATIONSHIP**

The decidual and trophoblastic tissues interact in an immunologically active environment in which there appear to be a number of conflicting demands placed on the maternal immune system. The need for surveillance against disease is the same for these tissues as for the rest of the body, however the unique presence of a tissue bearing foreign antigens residing within this setting seems to have led to the evolution of distinct immune cell populations with multi-purpose roles.

### **1.2.1 Overview of the immune system**

The immune system consists of leukocytic cells which are categorised by their function and by the surface CD (cluster of differentiation) markers they express. In any immune response two components are present; recognition of the foreign material and action to eliminate it. The mechanisms by which these actions are brought about fall into two main categories, many immune responses involve both types of response.

One arm of the immune system is an adaptive system, highly specific for a particular pathogen, involving the memory of previous encounters with the pathogen. This requires the production of antibodies against the pathogen and the response is more effective with subsequent exposure. The major cell types involved in this arm of the immune system are B lymphocytes, which synthesise antibodies; monocyte-macrophages which recognise foreign antigens then ingest and process them; and T lymphocytes which have a range of functions including stimulating B cells, retention of memory of pathogens and cytotoxicity.

The second method is non-specific and involves a faster but less focused approach. This defence process relies on the leukocytes identifying surface molecules on the target cells as foreign, then binding to and ingesting or lysing those cells. The major cell types involved in this process are the phagocytes, including polymorphonuclear leukocytes and

monocyte-macrophages which attack microorganisms; and natural killer cells which recognise tumour cells.

T cells recognise antigens after macrophages have ingested them, processed them and then presented peptides derived from them in association with major histocompatibility complex (MHC) molecules expressed on the surface of the macrophage. There are two classes of MHC antigens, class I which are expressed on all nucleated cells and are involved in recognition of non-self and class II which are only expressed on activated antigen-presenting cells such as macrophages and are involved in the recognition of pathogens.

An essential part of the processes described above is the secretion by leukocytes of cytokines. These are polypeptide molecules which signal between cells. They have a range of functions, most of which activate or regulate aspects of the immune system and provide a communication system which is involved with every aspect of the immune response.

### **1.2.2 Leukocytic cells present in the endometrium.**

In non-pregnant endometrium leukocytes are a major cell population comprising up to 50% of the total cell numbers. Leukocytes are found scattered in the stromal tissue and as cell aggregates (*Bulmer et al., 1991*) and in an intraepithelial position (*Kamat & Isaacson, 1987*). In proliferative endometrium there are three major populations of stromal leucocytes: CD56+ CD3- CD16- lymphocytes (decidual large granular lymphocytes), CD14+ macrophages and CD3+ T lymphocytes.

The number of endometrial leukocytes remains fairly constant through the proliferative phase and early secretory phase of the cycle but in the late secretory phase the number of stromal leukocytes increases mostly due to an increase in the number of CD56+ CD3- CD16- lymphocytes (*Bulmer et al., 1991*). This increase is probably in part due to proliferation, since cells observed in pre-menstrual endometrium are often in mitosis

(*Bulmer et al., 1991*). Leukocytes continue to make up a significant proportion of the cell population of the decidua. In early pregnancy up to 40% of the cells in the decidua are positive for the leukocyte marker CD45 (*Bulmer, 1988; Bulmer & Sunderland, 1984*).

#### **1.2.2.a Decidual large granular lymphocytes**

By the time of implantation the decidua contains a significant population of natural killer (NK)-like cells, decidual large granular lymphocytes (LGLs), the only known non-pathological situation in which NK cells are found in large numbers in tissues. The rapid rise in their numbers at this time is in part due to proliferation and part due to infiltration (*Bulmer et al., 1991*).

These cells bear an unusual phenotype found in less than 1% of the peripheral blood lymphoid cell population (*Lanier et al., 1986*) and they appear to be an organ-specific subset related to but distinct from classic circulatory NK cells which are large granular lymphocytes (LGLs). They have been termed decidual LGLs because of this resemblance. They stain intensely positive for CD56, which is an NK marker weakly expressed by peripheral blood NK cells, but do not stain for other NK markers such as CD16, CD57 and CD11b. They have cytoplasmic granules that contain perforin and granzyme A, cytolytic molecules indicating that these cells are potentially capable of killer activity. Suspensions of decidual cells enriched with these cells have been shown to have cytotoxic activity against the NK target cell K562, although this activity is lower than that seen with peripheral NK cells.

In the first trimester these cells represent 50% of the decidual leukocytes but their numbers decline in the second trimester (*Bulmer & Sunderland, 1984; Bulmer et al., 1988a*) until they are undetectable at term. They are present throughout the decidua but aggregate particularly around endometrial glands and arterioles (*Bulmer et al., 1991*). They are also found associated with the trophoblast - the regions of the decidua proximal to fetal tissue have high concentrations of these cells.

From their cell surface markers decidual LGLs appear to be highly activated in comparison to circulating NK cells, and have a pattern of activation marker expression characteristic of interleukin-2 (IL-2) stimulated NK cells.

### **1.2.2.b Macrophages**

Macrophages are a major cell population in the uterus and placenta throughout pregnancy making up 25% of the total leukocytic cell population (*Hunt et al., 1984; Lessin et al., 1988*). These cells are identified by their positive staining for the monocyte-macrophage marker CD14+ (*Bulmer & Johnson, 1984; Kabawat et al., 1985*) and are often associated with extravillous trophoblast (*Bulmer et al., 1988a; 1988b*) indicating a possible interaction with fetal tissue during and after implantation rather than a passive presence. These macrophages do not express CD15 - a marker of blood macrophages - and therefore seem to be a specific cell population distinct from monocyte-macrophages found in the peripheral circulation. Most of these cells stain intensely for the class II MHC antigens (*Sutton et al., 1983; Bulmer & Johnson, 1984*) and many express CD11c. The expression of these markers indicates that they are in an activated state (*Robbins et al., 1988*), are capable of secreting cytokines (*Nathan, 1987; Hunt, 1989*) and could be involved in antigen presentation to T lymphocytes. Isolated decidual macrophages are capable of antigen presentation in mixed lymphocyte reactions (MLRs) with cells from pregnant women as responder cells and their partners cells as stimulators (*Mizuno et al., 1994*). They can be further stimulated by endotoxin or gamma interferon ( $\gamma$ -IFN) from lymphocytes to release interleukin -1 (IL-1) and tumour necrosis factor (TNF) (*Hill & Anderson, 1988*), both of which are products of macrophages involved in the up-regulation of inflammatory responses.

In addition to releasing pro-inflammatory cytokines, macrophages are also a source of two immunosuppressive molecules, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and transforming growth factor-  $\beta$  (TGF- $\beta$ ). Both of these molecules suppress the proliferation of lymphocytes (*Lala et al., 1988; Matthews & Searle, 1987*) and the generation of activated killer cells.

Decidual macrophages contain enzymes, hydrogen peroxide and free radicals suggesting a phagocytic role (*Bulmer & Johnson, 1984*), perhaps involving the destruction of invading organisms (*Redline & Lu, 1988*). Phagocytosis in the decidua may also be required to remove tissue debris present as a by-product of the tissue remodelling that takes place after the invasion of the decidua by the trophoblast.

#### **1.2.2.c CD3+ T lymphocytes**

A third group of leukocytes found in the decidua are lymphocytes which stain positive for the lymphocyte marker CD3. Most of these cells additionally stain positive for CD8, identifying them as members of the cytotoxic subset of T lymphocytes involved in MHC class I interactions. These cells may be conventional T lymphocytes involved in interactions with macrophages and memory of pathogens rather than specific pregnancy related functions. However this group of cells is also capable of suppressor activity (*Bulmer, 1989*) and may be another cell type involved in creating an immunosuppressive environment in the decidua.

#### **1.2.2.d B lymphocytes**

There are very few B-lymphocytes in the endometrium or decidua and they are unlikely to play a significant role in the process of pregnancy.

### **1.2.3 Cytokines in the endometrium and decidua**

Many cytokines have been identified as present in the endometrium and decidua and their role is not confined to activities solely concerned with the immune system. Table 1.1 lists some of these cytokines and their proposed roles in the uterine environment.

#### **1.2.3.a Pro-inflammatory cytokines**

Several of the cytokines involved in the upregulation of inflammatory responses have been described in the endometrium and decidua. These include IL-1, TNF,  $\gamma$ IFN and IL-6. The release of any of these molecules may initiate a cascade reaction resulting in the

CYTOKINE	CELLULAR ORIGIN	ACTIVITY	PROPOSED ROLE IN ENDOMETRIUM OR DECIDUA	REFERENCE
IL-1	Activated macrophages	T-cell activator, induces IL-2 secretion, pyrogen, inflammatory reaction stimulator, induces PGE <sub>2</sub> release.	Regulation of hormones, inflammatory reaction against infection, menstruation	(Tabibzadeh, 1990)
IL-2	Activated T lymphocytes	Activates NK cells	Can cause trophoblast cytolysis by activating NK cells	(Parhar et al., 1989)
IL-3	Activated T lymphocytes	Multi-purpose growth factor	Growth promoter	(Armstrong & Chaouat, 1989)
IL-6	Macrophages, fibroblasts	Mediator of inflammation	Local signalling between hormones and cytokines	(Tabibzadeh et al., 1989)
GM-CSF	Activated T-cells	Growth promoter	Growth promoter	(Chaouat et al., 1990)
CSF-1	Macrophages, fibroblasts, endothelial cells	Growth promoter	Growth promoter	(Arceci et al., 1989)
$\gamma$ -IFN	Activated T-cells, NK cells, macrophages	MHC class I and II induction, NK cell stimulation, anti viral,	May cause trophoblast antigen recognition by upregulating MHC class I	(Hill, 1992)
TNF- $\alpha$	Activated macrophages, activated T lymphocytes	IL-2 receptor induction, $\gamma$ -IFN production, cytotoxic proinflammatory	Toxic to sperm, trophoblast, involved in menstruation	(Philippaiaux & Piguat, 1993)
TGF- $\beta$	Macrophages	Inhibitory and stimulatory	Immunosuppression, growth modulator	(Tabibzadeh, 1994)

**Table 1.1:** Cytokines in the endometrium and decidua and their proposed functions

secretion of the others and additional cytokines not usually found in large quantities in the decidua such as interleukin 2 (IL-2).

In non-pregnant endometrium IL-1 and TNF- $\alpha$  are found during the late secretory phase (*Tabibzadeh, 1990; Philippaeaux & Piguet, 1993*). The process of menstruation at the end of a non-conception cycle is reminiscent of an inflammatory reaction (*Tabibzadeh, 1994*) and IL-1 and TNF- $\alpha$  may be mediators of this process. Prostaglandins are involved in the onset of menstruation (*Singh et al., 1975*) and IL-1 increases PGE<sub>2</sub> synthesis by the epithelial cells of the endometrium (*Tabibzadeh, 1990*) providing a pathway by which cytokines may interact with the endometrium. In decidual tissue IL-1 levels rise during early pregnancy (*Kauma et al., 1990*) released by stromal cells and macrophages.

IL-2 is released by activated lymphocytes and stimulates the release of IL-1, TNF- $\alpha$  and  $\gamma$ -IFN and activates natural killer cells. Decidual cells can be induced to secrete IL-2 *in vitro* (*Sato et al., 1990*) but *in vivo* the release of this molecule appears to be blocked and IL-2 is not normally found in measurable quantities in endometrial or decidual tissue (*Lala, 1989*).

IL-6 is secreted by endometrial stromal cells in response to IL-1, TNF or  $\gamma$ IFN and is a major mediator of inflammatory responses. IL-6 levels can be also be modulated by oestrogens providing a link between hormones and the cytokine network (*Tabibzadeh et al., 1989*).

Placental tissue contains high levels of interferons (IFNs) (*Chard et al., 1986; Duc-Goiran et al., 1986*), the function of this is unknown although receptors for  $\gamma$ IFN, which induces class I antigens, are also present (*Branca, 1986*).

### **1.2.3.b Growth-modulating cytokines**

Colony-stimulating factor 1 (CSF-1) and GM-CSF are secreted by the epithelial cells of the endometrium in the peri-implantation period (*Arceci et al., 1989; Robertson et al., 1992*). These factors stimulate DNA synthesis in murine trophoblast cells in culture although they may not stimulate trophoblast cell proliferation (*Drake & Head, 1994*), and if administered to pregnant mice they reduce the fetal reabsorption rate and increase the placental weight observed (*Chaouat et al., 1990*). However GM-CSF also appears to suppress the growth of the trophoblast-like cell line Jeg-3. IL-3 is secreted by activated lymphocytes and stimulates the growth and differentiation of a broad range of cells. *In vitro* studies show IL-3 has only a mild stimulatory effect on the growth of trophoblast cells (*Drake & Head, 1994*). The three cytokines may be involved in growth promotion and differentiation of trophoblast cells but the extent of their effect is still to be fully identified.

TGF- $\beta$  is involved in the endometrial tissue growth and remodelling that occurs during the proliferative phase of the menstrual cycle. It modulates epithelial cell proliferation and enhances gland formation. It also promotes the activation and proliferation of fibroblasts and the deposition of extracellular matrix (*Tabibzadeh, 1994; Sporn & Roberts, 1990*), both processes are required for the regrowth of the stromal matrix. Another important attribute of TGF- $\beta$  is its potent immunosuppressive activity. The secretion of TGF- $\beta$  by decidual macrophages may be an important component of the mechanisms which regulate and prevent IL-2 release in the decidua.

### **1.2.4 The role of leukocytic cells in the endometrium**

The presence of leukocytic cells in the endometrium and early pregnancy decidua is not due to a passive infiltration but the result of an active recruitment process of specific cell populations. It can therefore be assumed that these cells have a role to play in the establishment and maintenance of successful pregnancy. There have been a number of theories put forward to explain the presence of these cells.



#### **1.2.4.a Surveillance**

One likely role these cells fulfil is that of surveillance. The female reproductive tract is exposed to several types of foreign antigens including microorganisms, sperm and fetal tissue. Although the uterus is a normally sterile environment with the cervix as a protective barrier (*Ansbacher et al., 1967; Larson & Galask, 1982*) there are occasions when it is invaded by bacteria such as listeria or viruses such as rubella, therefore the presence of immunologically active cells may be a pre-emptive guard against the possibility of infection. Decidual macrophages and the CD3+ T lymphocytes are probably involved if infection occurs.

Sperm can be seen as invading cells, but although they are antigenically foreign to the female they are able to pass to the fallopian tube to fertilise the ovum, and some at least are not eliminated by the maternal leukocytic cells. After intercourse there is a massive influx of leukocytes into the female cervix, the function of which may be to remove abnormal sperm, microorganisms and debris (*Thompson et al., 1992*). Decidual macrophages with their phagocytic ability may continue this process in the uterine cavity.

#### **1.2.4.b Recognition**

Another role of decidual leukocytic cells may be antigen presentation, to signal the presence of the fetus to the maternal immune system and induce recognition. This may be an important step in provoking a muted maternal-anti-fetal response which then gives a subsequent advantage to the fetus (*Edwards et al., 1985; Sutton et al., 1986, Lessin et al., 1988*), by the induction of blocking antibodies or the release of growth factors.

It appears that the trophoblast has developed a system for allowing maternal recognition to occur without activating a cascade of immune reactions that may result in cytolytic responses. There may however be occasions when this immunological balance breaks down and the trophoblast cells do come under attack.

In 1953 Medawar proposed that the fetus was a semi-allograft analogous to a transplant. This led to many studies investigating the expression by trophoblast cells of the MHC antigens involved in the recognition and rejection of transplants. The expression of these molecules by trophoblast depends on the anatomical location of the cells, the stage of differentiation of the cells and whether they are exposed to maternal or fetal blood.

Preimplantation embryos do not express class I MHC antigens (*Desoye et al., 1988*). Syncytiotrophoblast which is exposed to the maternal blood also does not express class I antigens (*Faulk & Temple, 1976; Goodfellow et al., 1976*) and express low to undetectable levels of class I mRNA (*Hunt & Hsi, 1990*). Cytotrophoblast within the villi which is not exposed to any maternal tissue contains class I mRNA (*Hunt & Hsi, 1990*) but the antigen is not expressed *in situ* (*Sunderland et al., 1981; Bulmer & Johnson, 1985*), although if the tissue is released from the villous structure and cultured the cells do express class I proteins (*Loke & Burland, 1988*). Extravillous trophoblast which proliferates and invades deep into the maternal decidua contains class I mRNA and expresses the antigen (*Sunderland et al., 1981*). This antigen was later shown to be expressed on chorion membrane as well (*Hsi et al., 1984*). None of the trophoblast cell populations express class II MHC antigens (*Loke, 1989; Faulk & McIntyre, 1983*), usually only expressed by leukocytic cells.

The class I antigen expressed by the extravillous trophoblast is a non-classical molecule, with a small heavy chain (*Ellis et al., 1986*) and has been identified as HLA-G by *in situ* hybridisation and by purification of first trimester cytotrophoblast cells (*Kovats et al., 1989*). Cultured cytotrophoblast cells can be induced to express class I antigens by  $\gamma$ IFN *in vitro* (*Loke & Burland, 1988; Feinman et al., 1987*). This make trophoblast cells susceptible to recognition when in an environment which contains  $\gamma$ IFN and antigen-presenting cells. Under these conditions there is a possibility that, *in vivo*, class I antigens are induced and that cytolysis follows the recognition of the trophoblast cells.

Cells from first trimester decidua have been shown to act as accessory cells for mitogen induced lymphoproliferation and the presentation of antigens to T cells (*Oksenberg et al., 1986*). The MHC II positive activated macrophage cells are prime candidates for this role.

This evidence demonstrates that there is the possibility of recognition of fetal antigens by maternal leukocytes in the decidua. The reasons for this and the mechanisms that control the process are still unclear.

#### **1.2.4.c Growth stimulation**

The theory of immunotrophism was originally proposed by Wegmann and co-workers (*Athanassakis et al., 1987*). The basis of this hypothesis is that there is an advantage for the feto-placental unit to be in close proximity to maternal leukocytes. It is suggested that an interaction between the fetal membranes and the leukocytes provoke a recognition response stimulating the leukocytes to release growth factors including interleukin-3 (IL-3), GM-CSF and CSF-1. These cytokines then stimulate the development of the fetus and the placenta. This idea is supported by experiments that demonstrate that depletion of maternal T cells reduces placental cell proliferation in placental-trophoblast co-cultures as measured by <sup>3</sup>H-Thymidine and that some of these growth factors stimulate murine trophoblast growth in culture (*Athanassakis et al., 1987*).

#### **1.2.4.d Growth regulation**

The most likely function of the LGL cells in human decidua is a role in control of trophoblast invasion (*King et al., 1993*). The LGLs are candidates for this role because of their proximity to the regions of trophoblast invasion. These cells are able to kill trophoblast cells *in vitro* (*Parhar et al., 1989*), although *in vivo* there is little evidence of trophoblast cell lysis. Decidual LGLs contain cytolytic enzymes including perforin, and granzyme A, both of which are involved in the induction of apoptosis (*Shiver et al., 1992*). Based on this observation it has been proposed that the mechanism by which the

decidual LGLs lyse aberrant trophoblast cells is through the release of these molecules and the induction of apoptosis (*King et al., 1993*), a process which can occur with little histological evidence (*Ellis et al., 1991*).

Further evidence for the theory of LGL involvement in trophoblast control is the observation that trophoblast invades more deeply into ectopic sites lacking decidual LGLs (*King et al., 1993*). When an embryo implants in the fallopian tube a patchy decidualisation reaction takes place in the tube with LGLs only found in these areas, but the embryo invades beyond these regions. The main immune reaction at the ectopic site resembles an inflammatory response, while decidualisation still occurs in the endometrium, despite the remoteness of the embryo.

Decidual macrophages are another pregnancy-specific cell group that may be involved in the regulation of trophoblast growth. They are capable of the secretion of potent immunosuppressive molecules such as TGF- $\beta$  and PGE<sub>2</sub> and are also clustered round areas of trophoblast invasion. These molecules may have a direct role in the suppression of trophoblast growth, or they may be part of a control loop that suppresses overactive LGLs preventing a cascade inflammatory reaction from occurring.

### 1.3 MECHANISMS OF FETAL PROTECTION

The evidence shows that there are a large number of activated maternal leukocytic cells in close proximity to the fetal-placental tissues. They are capable of recognising the trophoblast cells and under certain specific conditions of lysing and killing them, a process which may be a feature of normal pregnancy. However their presence also poses a considerable threat to the foetus. Various mechanisms have been proposed to explain how this potentially hostile environment is controlled to prevent any attack and there is the possibility that the breakdown of control mechanisms provides an explanation for recurrent miscarriage and other fertility problems.

#### 1.3.1 Immunosuppression

Decidual tissue has been shown to contain potent immunosuppressive molecules (*Bolton et al., 1987; Bulmer et al., 1991*). This activity is usually attributed to TGF- $\beta$  (*Clark et al., 1988*). Although decidual macrophages are a potential source of TGF- $\beta$ , other cells have been described as responsible for its secretion. Clark and co-workers proposed that the mechanism to protect the fetus from leukocytes involves suppressor cells (*Daya et al., 1985*). In mice some of these cells have been shown to be small non-T, non-B granulated lymphocytes which release a soluble factor related to TGF $\beta_2$  which blocks responses to IL-2 (*Clark et al., 1988*).

Suppressor cells have been found mice in the endometrium after ovulation and in the decidua of successful pregnancies. In mice with a deficiency of these cells there is a high abortion rate (*Clark et al., 1986*) and in human IVF and embryo transfer there is a mononuclear infiltrate deficient in suppressor cells in the endometrial tissue removed after failed pregnancies (*Daya et al., 1985*). The human equivalent of Clark's suppressor cells may be the NK-like decidual LGLs (*Bulmer, 1988; Bulmer et al., 1991*) but NK-like cells have been found in murine decidua (*Croy et al., 1985*) and seem to be a different population from the small and large suppressor cells. There is little evidence of human decidual LGLs exhibiting immunosuppressive activity. A more likely human

equivalent is the decidual lymphocytes which stain positive for CD3 and CD8. These cells are theoretically capable of suppressor activity but this has not yet been demonstrated, nor is it known whether they secrete TGF- $\beta$ .

There have been reports of suppression of mixed lymphocyte reactions by the supernatants of endometrial epithelial cell cultures. (*Bulmer et al., 1991*). It is possible that the immunosuppression observed in decidual lymphocyte preparations may in fact be due to contaminating epithelial cells (*Bulmer, 1988*). Semi-purified suppresser cells from human decidua show lower levels of suppression of mitogen-induced lymphocyte proliferation than unfractionated decidual cell suspensions, so they are not responsible for the total immunosuppressive activity found in decidual tissue extracts (*Bulmer, 1988*). Separation of the various cell types appear to show that no single cell population is responsible for the immunosuppressive activity of decidual tissue.

The maintenance of an IL-2-free environment in the decidua is important for fetal survival (*Lala, 1989*). This is because the activation of T cells, NK-like large granular lymphocytes, lymphokine activated (LAK) cells, and macrophages is a threat to the fetus, and is IL-2 dependent. When IL-2 is injected into pregnant mice it causes pregnancy failure (*Tezabwala et al., 1989*). LGLs are able to lyse trophoblast cells after exposure to IL-2, and the release of  $\gamma$ IFN, which is stimulated by IL-2, can induce the expression of MHC class I antigens on trophoblast (*Feinman et al., 1987*), permitting their recognition. Therefore the activation of an inflammatory response by IL-2 would initiate a range of potentially damaging processes that may lead to cytolysis of the trophoblast cells.

TGF- $\beta$  suppresses the generation of IL-2 activated killer cells, cytotoxic T cell activity and IL-1 dependent T cell proliferation, and its presence in the endometrium may prevent these IL-2-dependent reactions from occurring. PGE<sub>2</sub> also inhibits several immunostimulatory activities including mitogen proliferation of T cells, activation of NK

cells and generation of lymphokine activated killer (LAK) cells (*Lala, 1989*) and may contribute to the maintenance of an IL-2-free environment.

Other molecules implicated in immunosuppressive activity in the endometrium include progesterone,  $\alpha$ -feto protein, and chorionic gonadotrophin (hCG). Of these only progesterone is widely accepted as having true immunosuppressive activity.

Supernatants of IVF embryos suppress lymphocyte proliferation (*Daya & Clark, 1986*) however IVF medium alone has been shown to have immunosuppressive activity and the presence of immunosuppressive activity does not correlate with successful implantation of these embryos. One of the molecules responsible may be early pregnancy factor, a protein that is still ill-defined and is measured by the inhibition of lymphocyte rosette formation (*Morton et al., 1974; Shu-Xin & Zhenm-Qun, 1993*).

There is clear evidence for immunosuppression in decidual tissue and TGF- $\beta$  and PGE<sub>2</sub> are probably responsible for some, but not all of this activity. The other contributing factors have not yet been totally characterised. The immunosuppressive activity released by epithelial cells observed by Bulmer (*1988*) may be due to placental protein 14 (PP14), the subject of this thesis. This will be discussed in more detail in section 1.5.

### **1.3.2 Blocking antibodies**

The theory of blocking antibodies is based on the idea that during pregnancy the mother secretes antibodies that block the response between maternal leukocytes and paternal antigens, protecting the fetus (*Rocklin et al., 1976; 1982*). These antibodies are demonstrated using mixed lymphocyte cultures (MLC) with male lymphocytes as stimulator cells and maternal lymphocytes as responder cells. This reaction can be inhibited by maternal plasma or serum. However, these antibodies are not a clinical index of successful pregnancy, and blocking activity is not always detected in the serum of women with normal pregnancy, therefore they do not appear to play a significant role in the course of normal pregnancy.

### 1.3.3 TLX

The concept of TLX (trophoblast-lymphocyte-cross-reacting) antigens was originally proposed by McIntyre and Faulk (*Faulk et al., 1978*). This was based on the observation that a polyclonal antibody raised against a set of trophoblast antigens also recognised antigens on lymphocytes and some other cell types including monocytes, PMNLs, and a weak interaction with platelets. (*Johnson et al., 1981; Bulmer et al., 1984; Bulmer & Johnson, 1985*). These antibodies can inhibit *in vitro* mixed lymphocyte reactions (*McIntyre & Faulk, 1979*). The same antigens are found in the serum of some patients suffering recurrent miscarriages. The theory is that the TLX antigen allows the trophoblast cells to participate in the local immune environment (*Bulmer, 1988*) maybe by stimulating the mother to mount an immune response which is beneficial to fetal development, and this explains both the role of immune cells in the endometrium and why they do not pose a threat to the foetus. The significance of these antigens has not been satisfactorily established and their relevance in spontaneous miscarriage is still controversial. It has also been suggested that successful pregnancy depends on a balance between TLX-Ag and blocking antibodies.



## 1.4 MODELS OF PREGNANCY FAILURE

Only 30% of embryos survive to birth, of the 70% that do not survive 15% end in recognisable miscarriage and 55% are lost in the early stages (*Lindley, 1979*). 91.7% of the early stage losses are without the mothers knowledge (*Edmonds et al., 1982*).

Although a high number of these early pregnancy losses are attributable to chromosomal abnormality (30-60%), the remaining 40-70% of embryo mortality is not due to grossly abnormal karyotype (*Flamigni et al., 1991*). There have been a number of theories put forward to explain this wastage.

### 1.4.1 Endometrial, oocyte and hormonal factors

It has been proposed that implantation is the crucial event which differentiates fertile and non-fertile cycles, (*Navot et al., 1989*) and that endometrial receptivity is the most important factor differentiating between successful and unsuccessful implantation (*Flamigni et al., 1991*). Data from *in vitro* fertilisation (IVF) supports this view; a 70-90% fertilisation rate results in only a 15-25% pregnancy rate (*Navo et al., 1986*) despite the replacement of multiple embryos. On average only 3-5% of embryos transferred after IVF successfully complete implantation (*Flamigni et al., 1991*), the rates of spontaneous miscarriage for IVF patients are higher than average and this reduces the live birth rate to below 10%. The timing of embryo replacement could be critical, as the endometrium may be hostile to out of phase embryos (*Edwards et al., 1981*). This is an important consideration in IVF, since embryos that are fertilised outside the body, cultured *in vitro*, and replaced develop more slowly than in the normal *in vivo* situation and therefore no longer match the dating of the endometrium into which they are replaced.

The drop in fecundity with age has been attributed by some to uterine ageing, and others to oocyte ageing. With advances in assisted reproductive technology these theories have been tested in oocyte donation programmes. The results are ambiguous with some

studies demonstrating uterine age as a parameter in successful pregnancy (*Flamigni et al., 1993; Meldrum, 1993*). Other studies report a restoration of fertility in older women when donor eggs are used, independent of the age of the recipients (*Abdalla et al., 1993; Navot et al., 1994*). Both factors are likely to play a role and the balance may vary between different women.

Hormonal imbalance and endometrial retardation (histological dating behind LH dating by 2 or more days) are major factors in the aetiology of recurrent miscarriage (*Horta et al., 1977*). Recurrent miscarriage is defined as three or more consecutive fetal losses before the gestational age of 20 weeks and 15% of all pregnancies are lost through miscarriage spontaneously therefore 3-4 out of every 1000 women will have 3 or more consecutive losses by chance alone. Even in these patients the chance of success in the next pregnancy is 40-70%. This makes it very hard to assess the efficacy of any treatment. Hormonal therapy can improve the pregnancy outcome in these patients but emotional support and counselling can be equally as effective (*Stray-Pederson & Stray-Pederson, 1988*). A combination of the two can restore the probability of successful pregnancy to that of the normal population i.e. 85%, in the majority of patients (*T.C. Li, personal communication*).

#### **1.4.2 Blocking factors**

An absence of blocking antibodies has been observed in recurrent miscarriage (*Rocklin et al., 1976*). But the lack of antibodies may be the result of the pregnancy failure not the cause. Immunotherapy has been carried out as a treatment for recurrent miscarriage based on the theory that blocking antibodies must be induced to block a maternal-anti-fetal immune response. This treatment involves the immunisation of the patient with paternal (*Beer et al., 1985*) or third-party (*Taylor & Faulk, 1981*) leukocytes. A variation is immunisation with trophoblast vesicles (*Johnson et al., 1988*). Although success rates of up to 78% have been reported, the placebo effect of entering a trial and having careful monitoring and medical attention must not be underestimated (*Stray-Pederson & Stray-Pederson, 1988*). In addition some of these trials were restricted to

secondary recurrent miscarriage patients, based on the assumption that these were the most likely group to be suffering from a reaction to their partners antigens rather than their secondary recurrent miscarriage being due to another cause. While this is a logical step to take in the design of such a trial it further complicates analysis of the efficiency of the treatment and comparisons with the general population of recurrent miscarriers may imply that the treatment is more successful than it actually is. Until the results from a number of controlled trials currently being carried out are known the role of blocking factors remains controversial.

#### **1.4.3 Immunological imbalance**

There may be an immunological imbalance in recurrent miscarriage patients, either the leukocytes of the decidua are over-activated and prevent implantation or initiate rejection of the fetus or the natural immunosuppressive factors may be absent (*Hill, 1990*).

In spontaneous miscarriage the decidua shows large areas of necrosis and the fetal tissue is infiltrated by cytotoxic T lymphocytes (*Croy et al., 1982*), the cells which are activated by IL-2. These cells may have been activated due to a rise of cytokines in response to immunological imbalance, clinical or sub-clinical infection such as *Listeria* or because of recognition of the paternal antigens expressed by the fetus. In humans the presence of raised levels of TNF or IL-2 at the time of implantation has detrimental effects on the embryo (*Hill et al., 1987; Tezabwala et al., 1989*) and can cause miscarriage. In mice these cytokines can totally block pregnancy. However it is difficult to demonstrate that the cytotoxic cells are the cause of the fetal demise, their presence may only be a consequence of another mechanism already in process. In humans inevitably the evidence is only available after both cause and effect have already taken place.

The concept of immunologically mediated miscarriage is still controversial. However the evidence points to a system of immunological balance between activated leukocytes and immunosuppressive mechanisms during successful pregnancy. The breakdown of this system is likely to account for fertility problems in some women.

## 1.5 PLACENTAL PROTEIN 14

Placental protein 14 (PP14) is the major secretory product of the endometrium during the secretory phase of the menstrual cycle and the first trimester of pregnancy, by the 7th week of gestation PP14 forms 10% of the total soluble protein extracted from the decidua (*Julkunen et al., 1985*).

### 1.5.1 Background

PP14 was first isolated by Bohn and colleagues (*Bohn et al., 1982*) and has since been shown to be structurally similar or identical to a number of other molecules discovered by different groups of workers. These include chorionic  $\alpha$ -2 microglobulin (*Petrinin et al., 1976; Petrinin et al., 1978*), alpha uterine protein (*Sutcliffe et al., 1980*), progestogen-dependent protein (*Joshi et al., 1980*), pregnancy associated endometrial  $\alpha$ -2 globulin and endometrial protein 15 (*Bell et al., 1985a; 1985b*). Bohn's original preparation was described as having a molecular weight of 42,000 daltons but this was later revised to 56,000 daltons made up of two identical subunits each having a molecular weight of 28,000 daltons (*Julkunen et al., 1988*). The isoelectric point of PP14 is 4.8 (*Julkunen et al., 1988*). The cDNA sequence of PP14 has been published (*Julkunen et al., 1988*), this shows that PP14 is coded for by a 1 kilobase mRNA and is made up of 180 amino acids including a hydrophobic rich 18 amino acid signal sequence. This gives a predicted molecular weight of 18,787 daltons for the mature protein. The carbohydrate content of PP14 is generally quoted as 17.5%, however this calculation appears to have been made using a molecular weight of 21,000 daltons for the subunits of PP14. Using the revised subunit weight of 28,000 daltons the carbohydrate content can be calculated as 33%.

### 1.5.2 PP14 is a lipocalin

PP14 is a member of a family of proteins that has been named the lipocalins (*Pervaiz & Brew, 1985*). The name comes from the greek word calyx meaning pocket and has been coined because all the members of the family so far identified have a binding pocket

which binds a small hydrophobic molecule. PP14 has 53.4% homology with equine  $\beta$ -lactoglobulin (*Bell et al., 1987; Huhtala et al., 1987*) and 22.7% homology with retinol binding protein (*Seppälä et al., 1988*). PP14 does not bind to retinol (*Westwood et al., 1988*) although  $\beta$ -lactoglobulin does (*Futterman & Heller, 1972*). Bohn originally proposed that PP14 is a prostaglandin metabolising enzyme (*Bohn et al., 1982*). However the secretion pattern of PP14 does not fit with this hypothesis. PP14 is secreted mainly during the late secretory phase of the menstrual cycle and the first trimester of pregnancy, a secretion pattern which doesn't correspond to that of prostaglandins, the levels of which gradually rise throughout pregnancy. Another possible ligand for PP14 is progesterone which is immunosuppressive and essential for pregnancy. The lipocalin family is reviewed in greater detail in the introduction to chapter 5.

### **1.5.3 PP14 in the reproductive tract**

PP14 is secreted from the epithelial cells of the glandular endometrium into the uterine luminal fluid (*Bell & Doré-Green, 1987*) PP14 is found in follicular fluid (*Seppälä, 1985*) fallopian tubes (*Julkunen et al., 1986a*), and amniotic fluid, where the concentration of PP14 at 8-10 weeks gestation is 100-1000 times that found in serum (*Julkunen et al., 1985*). In the male, seminal plasma has been found to contain high levels of PP14 up to 800 $\mu$ g/ml forming 2.5% of the total protein content (*Bell & Patel, 1987; Bolton et al., 1986*).

Serum levels of PP14 vary during the menstrual cycle in a pattern which reflects ovulation (*Joshi et al., 1982; 1986; Julkunen et al., 1986b*) There are basal levels of PP14 detectable throughout the cycle. This pattern reflects the development of the endometrium which is proliferating during the period leading up to ovulation and then secreting proteins in the second half of the cycle in preparation for the implantation of the fertilised ovum. In situ hybridisation studies show that mRNA for PP14 is detectable in the basal glands of early proliferative phase endometrium, virtually undetectable in the late proliferative phase and early secretory phase and abundant after day 17 of the

menstrual cycle, but only in the epithelial glandular cells (*Seppälä et al., 1988b*). PP14 mRNA was also detected in the fallopian tubes in the mucosal epithelial cells in the late secretory phase. Immunohistochemistry confirms this picture with PP14 first detectable from day 19-21 in epithelial glands, with the levels reaching a maximum at the end of the secretory phase (*Waites et al., 1988*). In conception cycles the levels of PP14 in serum rise dramatically, doubling every 2.5 days (*Joshi, 1987*), reaching a peak at 7-9 weeks then dropping after 8-10 weeks (*Bell et al., 1985a; Joshi et al., 1982*).

This pattern of secretion led to the assumption that PP14 is progesterone dependent (*Joshi et al., 1980*) as some of the synonyms imply. Oral contraceptives containing progesterone can cause an increase in PP14 (*Seppälä et al., 1987*), but this may only be an indirect effect. There is a 6 day gap between the rise in progesterone and that of PP14 which is unusual for a tissue release response to a hormone. There is no fall in PP14 when RU386, a progesterone receptor antagonist is administered (*Howell et al., 1989*). The levels of PP14 begin to fall after 9-10 weeks of pregnancy, at this time the levels of progesterone are still rising, although the drop in PP14 secretion may reflect the changes in the secretory pattern of the endometrium at this time. It now appears that PP14 secretion is dependent on oestrogen priming of the endometrium and ovarian hyperstimulation in IVF treatment causes a proportional rise in PP14 secretion.

#### **1.5.4 The role of PP14**

PP14 has been the focus of much interest and study to try and elucidate the role it plays in the endometrium. The clinical data are contradictory and lead to conflicting conclusions. Subjects with inadequate luteal responses as assessed by histology have low levels of PP14 (*Joshi et al., 1986*) and in IVF treatment there is an association between implantation and a rise in PP14 (*Than et al., 1988; Wood et al., 1990*). Yet PP14 does not appear completely essential for pregnancy since Turner's syndrome patients are able to carry pregnancies to term after embryo transfer and hormone supplement despite having normal levels of progesterone and subnormal levels of PP14 (*Johnson et al., 1993*). Similar findings have recently been reported in patients with

premature ovarian failure. These findings seem dependent on the hormone regime followed.

It has been proposed that PP14 has an immunosuppressive role in the protection of the feto-placental tissue from the potentially hostile maternal immune system. Decidual tissue extracts inhibit mixed lymphocyte reactions and the mitogen stimulated proliferation of lymphocytes (*Bolton et al., 1987; Pockley et al., 1988*). This activity can be removed using a specific monoclonal immunoadsorbent to PP14 and purified PP14 alone has the same activity. PP14 suppresses the release of IL-1 and IL-2 from mitogenically stimulated mononuclear cells (*Pockley & Bolton, 1989; 1990*). This may show that the key action of PP14 in suppressing lymphoproliferation is through the inhibition of the production and/or the release of IL-1.

PP14 has also been shown to suppress natural killer cell activity (*Okamoto et al., 1991*) an interesting observation in the light of the presence of the large number of NK-like large granular lymphocytes in decidual tissue during pregnancy. NK cells secrete IL-1 when activated and therefore the action of PP14 to suppress IL-1 release may not be restricted to monocyte-macrophages.

## 1.6 AIMS OF THE STUDY

The overall aim of this study was to investigate, from a number of perspectives, the possible role of PP14 as an immunomodulator involved in reproduction.

Chapter 1 has described the background context of the active immunological environment in which PP14 is found. A case has been made for the need of immunosuppressive molecules in this environment. PP14 has been suggested as a candidate molecule for this role.

The remaining chapters of this thesis describe studies which contribute to furthering the understanding of the role of PP14. These studies focus on three particular areas.

### 1.6.1 Purification and activity studies

Much of the work on the activity of PP14 has been carried out on crude decidual extracts (*Bolton et al., 1987*). Although the purification of PP14 has been reported (*Westwood et al., 1988; Riittinen et al., 1991*), the quantities obtained are of the order of a few milligrams. In addition the second method involves the use of acidic buffers which may denature the protein. To carry out further activity studies a method that yielded larger quantities of PP14 was desirable, provided the risks of denaturation were minimised.

The ability of PP14 to suppress the response of peripheral blood mononuclear cells to mitogen stimulation, in mixed lymphocyte reactions and in natural killer assays has previously been investigated. The cell stimulation that is the basis for all these assays is dependent on the response of the cells to IL-2. It is known that PP14 suppresses the release of IL-2 *in vitro* but its direct effect on the response to exogenous IL-2 is not known. This is an important physiological question since the activation of the NK-like cells in the decidua is dependent on their response to IL-2.



Previous *in vitro* studies on the activity of PP14 have involved primary cell cultures, of either peripheral blood mononuclear cells (*Bolton et al., 1987; Pockley et al., 1990*) or natural killer cells (*Okamoto et al., 1991*). The variability observed in these studies has been singled out for particular mention (*Pockley et al., 1990*). This variation is attributed to the use of blood samples from different donors taken on different days, an inherent problem in primary cell cultures. The study of certain aspects of PP14 has been hampered by this difficulty. Investigations into the stability of the activity, for instance, are hard to interpret when the natural variation from one experiment to the next may be higher than the activity changes under investigation.

Many assays that measure the activity of immunomodulatory molecules are based on the effect of that molecule on the growth of a cell line or on the release of another molecule by a cell line. For example, the activity of the immunosuppressive molecule transforming growth factor- $\beta$  is measured by its ability to suppress the growth of the mink lung cell line Mv-1-Lu (*Meagher et al., 1991*).

The development of a similar bioassay for PP14 using a cell line would eliminate many of the problems of primary cell assays and provide a reproducible way of relating the activity observed from one assay to the next.

### **1.6.2 Expression of PP14 as a recombinant protein and molecular modelling of PP14**

If PP14 is found to be an important immunomodulatory molecule in human reproduction there are two areas of study which also become significant. The first is the question of its use as a possible treatment. If PP14 has a role in normal pregnancy it is likely that a lack of it could result in problems, which the therapeutic administration of PP14 might resolve. Recombinant PP14 protein would be essential for therapeutic administration. Recombinant PP14 would also be an additional source of PP14 for other studies.

A second area of interest is the mechanism of action of the protein. PP14 is a member of the lipocalin family, a group of carrier molecules that bind small, hydrophobic molecules. The significance of this is unknown, as is the cofactor or ligand that PP14 binds. The molecular structure of some of the lipocalins is known, but it is not known how PP14 resembles these molecules. Insights into the structure of PP14 would help in identifying its mode of action. In addition, information about the identity of the cofactor may be essential for an active form of recombinant PP14 to be developed.

### **1.6.3 Clinical significance of PP14**

Many attempts have been made to ascertain the clinical significance of PP14. Almost all of these studies have relied on the measurement of PP14 in serum or plasma. It has generally been accepted that the results from these experiments are unlikely to accurately reflect the PP14 levels in the local environment of the endometrium or decidua.

If PP14 has an important role as an immunomodulator during pregnancy, a lack of this protein may be associated with unexplained infertility or recurrent miscarriage. A method of examining the local levels of PP14 may provide the opportunity of investigating the clinical relevance of PP14 in these conditions.

### **1.6.4 Summary of aims**

The studies described in this thesis had four aims:

1. To purify PP14 and use this to set up an activity assay.
2. To clone the cDNA coding for PP14 and express PP14 as a recombinant protein.
3. To model the structure of PP14.
4. To investigate the clinical relevance of PP14.

## CHAPTER 2: PURIFICATION OF PP14

### 2.1 INTRODUCTION

In order to carry out the activity studies described in Chapter 3 it was essential to have a source of pure, active PP14. Several methods have been published describing the purification of PP14, however these use reagents which risk denaturation of the protein (*Riittinen et al., 1991; Westwood et al., 1988*), or introduce reagents such as Concanavalin A (*Bell et al., 1987*) which may contaminate the preparation and affect later immunological assays.

Using the published methods as a starting point the aim of this study was to develop a purification which reduced the risk of denaturation but yielded sufficient quantities of active PP14 from each run (75-100 mg) at an acceptable level of purity (>95%).

## **2.2 MATERIALS AND METHODS**

All reagents, suppliers and equipment are listed in Appendix I.

### **2.2.1 Ethical aspects**

Approval for this study was obtained from the local ethics committee.

### **2.2.2 Purification strategy**

The method of Westwood et al. (1988) described an ion-exchange based purification procedure for PP14 using first trimester decidual tissue as the source material. Decidual tissue has the advantage that up to 10% of the total soluble protein content consists of PP14 (Julkunen et al., 1985). It is also easy to obtain in quantity. The tissue will be contaminated with blood, and so haemoglobin and albumin are major contaminants. Initial optimisation experiments established that up to 500µg of PP14 could be purified in one step on a Mono Q ion exchange column as used in the method of Westwood et al. (1988). The sample obtained ran as one band using SDS-PAGE gels. However this method did not provide enough material for functional studies so additional steps were incorporated to increase the capacity of the procedure. Based on the observation that crude decidual extract lost some immunosuppressive activity if left at room temperature for several hours, the protein preparation was kept at 4°C or below throughout the entire purification procedure. All the buffers were pre-cooled to 4°C and the columns were run with water chilled to 4°C passing through the outer water jackets.

### **2.2.3 Optimisation of the purification procedure**

#### **2.2.3.a Step 1**

A DEAE ion exchange column was incorporated as the first step, due to the high capacity of the gel (100mg total protein/ml gel) (*Pharmacia, technical information*). A pH of 7.2 was selected because this is the pI of haemoglobin, which would therefore pass through the column without binding, significantly reducing the total protein load required on subsequent columns and reducing the contamination of these columns. This step also

allowed a large volume of tissue extract (up to 4 litres) to be reduced to 500 ml in one step. Initial studies showed that the yield of PP14 after this step was reproducibly over 90% and the majority (over 90% by SDS-PAGE gel analysis) of the haemoglobin was removed. A NaCl concentration of 0.25M was chosen to elute the protein from the column. This concentration was decided from the results of the test run on the Mono Q column which indicated that PP14 was eluted at 0.15M, the concentration was increased by 0.1M to ensure that all the PP14 was recovered.

### **2.2.3.b Step 2**

The major contaminating protein remaining after the first ion-exchange step was albumin. Some of the properties of PP14 and albumin are similar, for example, the pI of PP14 is 4.5 and that of albumin 4.8 so a separation by isoelectric focusing would not separate them satisfactorily. In addition, the molecular weights are within 10,000 kD. One classic way to purify albumin is to use dyes to bind albumin. However, in a pilot experiment over 60% of PP14 was found to also bind to the dye substituted agaroses that bound albumin.

Hydrophobic interaction chromatography was used in the method of Riittinen et al. (1991) so this was explored as a possible step to separate PP14 from albumin. An optimisation experiment was carried out to investigate different hydrophobic resins. The binding of albumin to the resins was monitored using SDS-PAGE, the binding of PP14 was followed by measurement using the radioimmunoassay described in Appendix II. A 2 ml sample of the eluent from the DEAE step, containing 1.25 mg of PP14, was applied to 2ml test columns of each of the gels in phosphate buffer (pH 7.0, 10mM) containing 1M ammonium sulphate. If the sample bound it was then eluted by the following buffers in succession:

- (a) 10 mM phosphate, 1M ammonium sulphate
- (b) 10 mM phosphate
- (c) 50% isopropanol

The elution conditions and recovery of PP14 were noted for each column and compared to the elution conditions for albumin. The results are summarised in Table 2.1

From the results of this experiment hexyl agarose was chosen as the resin which was most likely to separate albumin and PP14 because albumin and PP14 both bound to it but could be eluted in non-denaturing conditions. When scaled up as described in the final method protocol separation between PP14 and albumin was observed.

### **2.2.3.c Step 3**

Next a gel filtration step was added which removed some further albumin along with high and low molecular weight contaminants. After the hydrophobic interaction step the sample volume was between 50 and 100mls. This sample was concentrated to reduce the volume before loading onto the gel filtration column. A Superdex 200 HR column was chosen because it combines a fast flow rate with high resolution, allowing a run to be carried out in 1.5 hours. 150mM NaCl was added to the buffer to prevent non-specific binding of proteins to the column.

### **2.2.3.d Step 4**

Finally the Mono Q step used in the method of Westwood et al. (1988) was replaced with a Hiload Q Sepharose ion exchange column with similar resolution but higher capacity (25mg total protein/ml gel). The flow rate and gradient volume of this final step were optimised to obtain a peak of pure PP14.

All the chromatography steps were carried out using Fast Protein Liquid Chromatography (FPLC) equipment from Pharmacia. At all stages in the purification procedure the fractions were assayed for their PP14 content using radioimmunoassay as detailed in Appendix II.

<b>RESIN</b>	<b>PP14 BINDING</b>	<b>PP14 ELUTION BUFFER</b>	<b>PP14 RECOVERY</b>
ETHYL	not bound	1M ammonium sulphate	100%
BUTYL	not bound	1M ammonium sulphate	85%
PROPYL	not bound	1M ammonium sulphate	100%
PENTYL	25% bound	1M ammonium sulphate/10mM Phosphate	100%
HEXYL	bound	10mM Phosphate	70%
OCTYL	bound	10mM Phosphate	52%
DECYL	bound	50% isopropanol	26%
DODECYL	bound	irreversibly bound	0%
<b>RESIN</b>	<b>ALBUMIN BINDING</b>	<b>ALBUMIN ELUTION BUFFER</b>	
ETHYL	not bound	1M ammonium sulphate	
BUTYL	not bound	1M ammonium sulphate	
PROPYL	not bound	1M ammonium sulphate	
PENTYL	25% bound	1M ammonium sulphate/10mM Phosphate	
HEXYL	75% bound	1M ammonium sulphate/10mM Phosphate	
OCTYL	bound	10mM Phosphate	
DECYL	bound	50% isopropanol	
DODECYL	bound	irreversibly bound	

**Table 2.1:** PP14 and albumin binding to hydrophobic resins. A protein sample after the initial ion exchange step was applied to each resin sample as detailed in the text and the binding, elution conditions and recovery of PP14 for each resin were determined. PP14 bound to hexyl agarose and could be eluted under non-denaturing conditions with resolution from contaminating albumin.

## **2.2.4 Final method protocol**

A flow diagram of the final purification scheme is shown in Figure 2.1.

### **2.2.4.a Collection and preparation of decidual tissue samples**

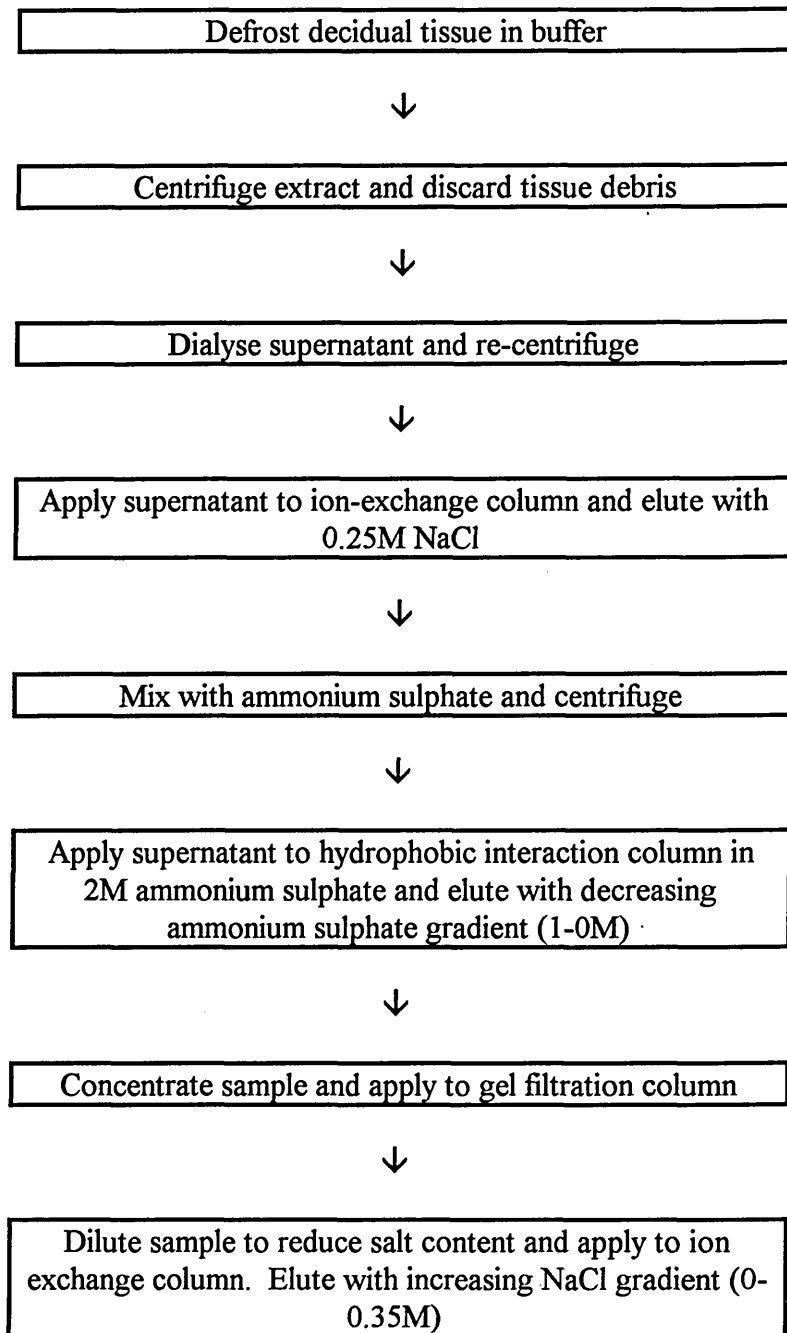
Decidual tissue was obtained from women undergoing elective termination of pregnancy, frozen immediately and stored at -20°C until used. The tissue was thawed overnight at 4°C in thawing buffer (20mM phosphate pH 7.2, 0.1mM dithiothreitol, 0.1mM ethylenediamine tetraacetic acid, 0.1mM phenylmethylsulphoxide). Dithiothreitol was added to stabilise disulphide bridges and retain protein folding, ethylenediamine tetraacetic acid was added to chelate metal ions required by metalloproteinases and phenylmethylsulphoxide was added as a proteinase inhibitor. The aim of the addition of these reagents was to protect the protein from denaturation.

Typically 250g of tissue was thawed in 3 litres of buffer. The freezing and thawing process had the effect of lysing the cells and releasing PP14 which is a secreted protein. Recovery of PP14 was not significantly improved if the tissue was homogenised, and contamination from other proteins increased. The mixture of thawed tissue and buffer was centrifuged (10,000g, 30 minutes, 4°C) and the supernatant passed through a funnel of glass wool to remove lipids. The supernatant was dialysed overnight at 4°C against thawing buffer to remove low molecular weight substances and to ensure the removal of any salt which would reduce the binding of the protein to the ion-exchange resin. Typically 500ml of supernatant was dialysed against 3 changes of 25 litres of buffer. Following dialysis the extract was re-centrifuged using the same conditions as before and stored at -70°C until required.

### **2.2.4.b Ion exchange chromatography**

Up to 2500 mls of decidual extract was applied at a flow rate of 5mls/minute to a 300ml column (15cm x 50mm) of DEAE Sepharose Fast Flow equilibrated in 20mM phosphate buffer pH 7.2 (buffer A). The column was washed with two column volumes of buffer A





**Figure 2.1:** Flow diagram of the final PP14 purification procedure

at the same flow rate and the bound protein eluted in one step by washing with buffer A containing 0.25M NaCl.

#### **2.2.4.c Hydrophobic interaction chromatography**

Saturated ammonium sulphate (4M, pH adjusted to 7.2)) was added to the eluted protein pool and then mixed for 30 minutes at 4°C on an end-over-end mixer to make a final total salt concentration of 1.5M. The mixture was centrifuged (10,000g, 30 minutes, 4°C) and the supernatant applied to a 300ml (15cm x 50mm) hexyl agarose column previously equilibrated with buffer A containing 1.5M ammonium sulphate at a flow rate of 5ml/minute. The column was then washed with two column volumes of equilibration buffer. A 1.5M ammonium sulphate concentration was found to be required to ensure all the protein bound to the column, but once it had bound no protein was eluted at ammonium sulphate concentrations between 1.5 and 1.0M. Therefore a gradient of 1M-0M ammonium sulphate was applied in 2 column volumes to elute the protein. The eluted protein was collected in 10ml fractions which were assayed for PP14 content and the peak fractions were pooled.

#### **2.2.4.d Concentration of pooled fractions**

The sample was concentrated using Amicon Centriprep-10 concentrators. Each concentrator had a capacity of 15mls. The pooled sample was centrifuged at 4°C for 40 minutes at 1500g. Typically a pool of 250ml would be concentrated to 25mls.

#### **2.2.4.e Gel filtration**

The concentrated PP14 was applied to a Superdex 200 HR column equilibrated with Buffer A containing 150mM NaCl. The sample was applied in 5ml aliquots and the experiment repeated for as many times as needed, typically 5 times. The flow rate of the column was 4ml/minute and 5ml fractions were collected. The peak fractions containing PP14 identified by radioimmunoassay were pooled from all the column runs.

#### **2.2.4.f Ion exchange chromatography**

The pool of PP14 fractions from the gel filtration was diluted with 4 volumes of water to reduce the salt concentration and was then applied at 5ml/minute to a 60ml Q Sepharose HiLoad column (2.6cm x 10cm) equilibrated with 5 volumes of buffer A. The column was washed with 2 column volumes of buffer A and then a gradient of 0-0.35M NaCl in 5 column volumes was applied. The PP14 peak was pooled.

#### **2.2.5 SDS-Polyacrylamide gel electrophoresis**

The purity of the PP14 was assessed by analysing samples using standard SDS-polyacrylamide gel electrophoresis (*Laemmli, 1970*) details of the method are in Appendix IV.

#### **2.2.6 Western blotting**

Western blotting was carried out on the SDS-PAGE gels. The blots were probed with polyclonal and monoclonal antibodies. The monoclonal antibody has previously been described as specifically immunoreactive with PP14 (*Bolton et al., 1987*). The details of this method are in Appendix IV. The polyclonal antibody was used at a dilution of 1/500 and the monoclonal at a dilution of 1/100. The monoclonal antibodies were subcloned, expressed and purified as detailed in Appendix III.

#### **2.2.7 Protein assay**

The total protein content of the samples obtained during the purification process was measured using a Pierce BCA protein assay kit. The details of this method are in Appendix V.

## **2.3 RESULTS**

The results detailed here are from a typical purification of PP14.

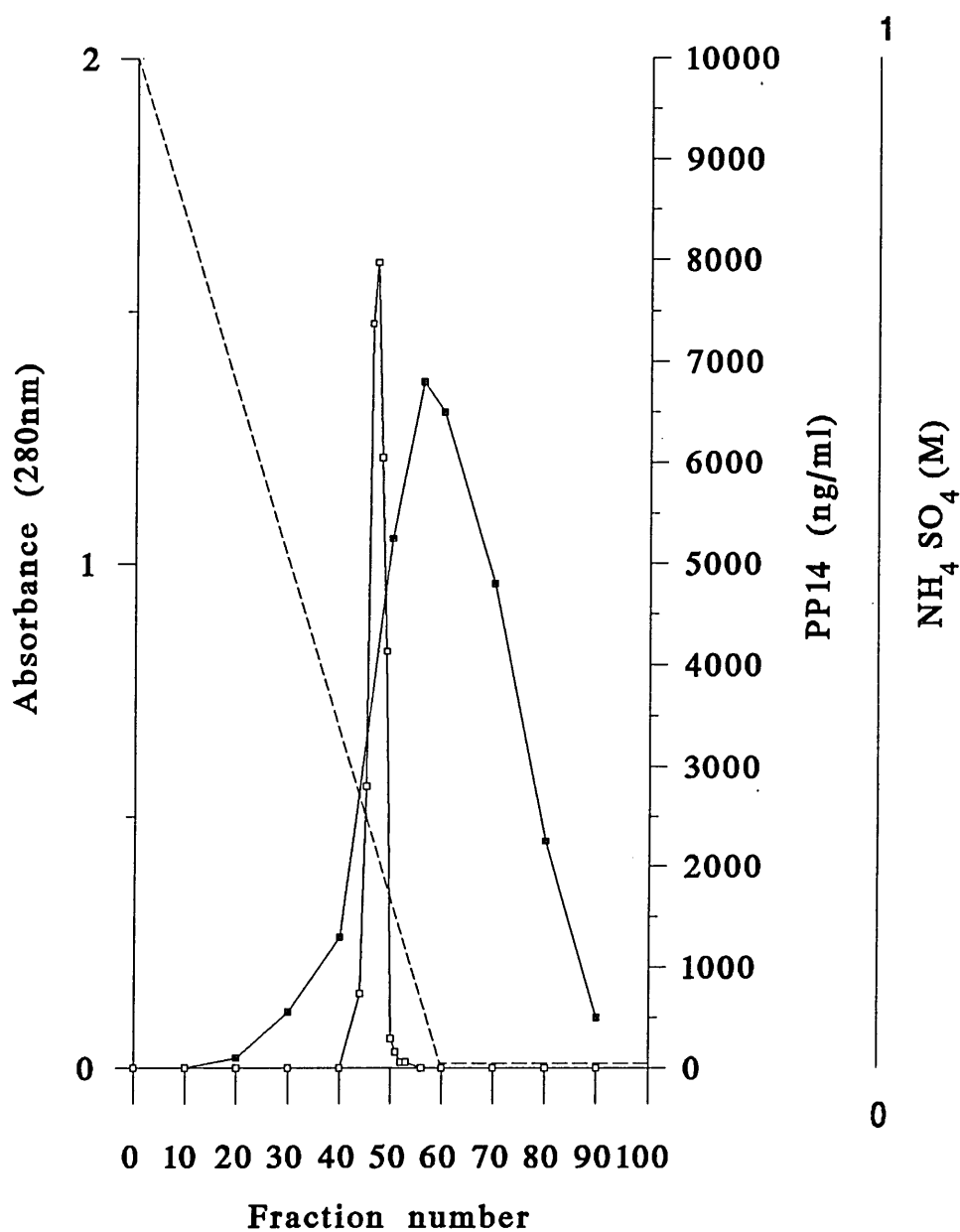
### **2.3.1 Chromatography steps**

The elution profiles from the hydrophobic interaction, gel filtration and ion exchange chromatography steps as identified by absorption at 280nm, and the PP14 concentrations measured by radioimmunoassay are shown in Figures 2.1, 2.2 and 2.3.

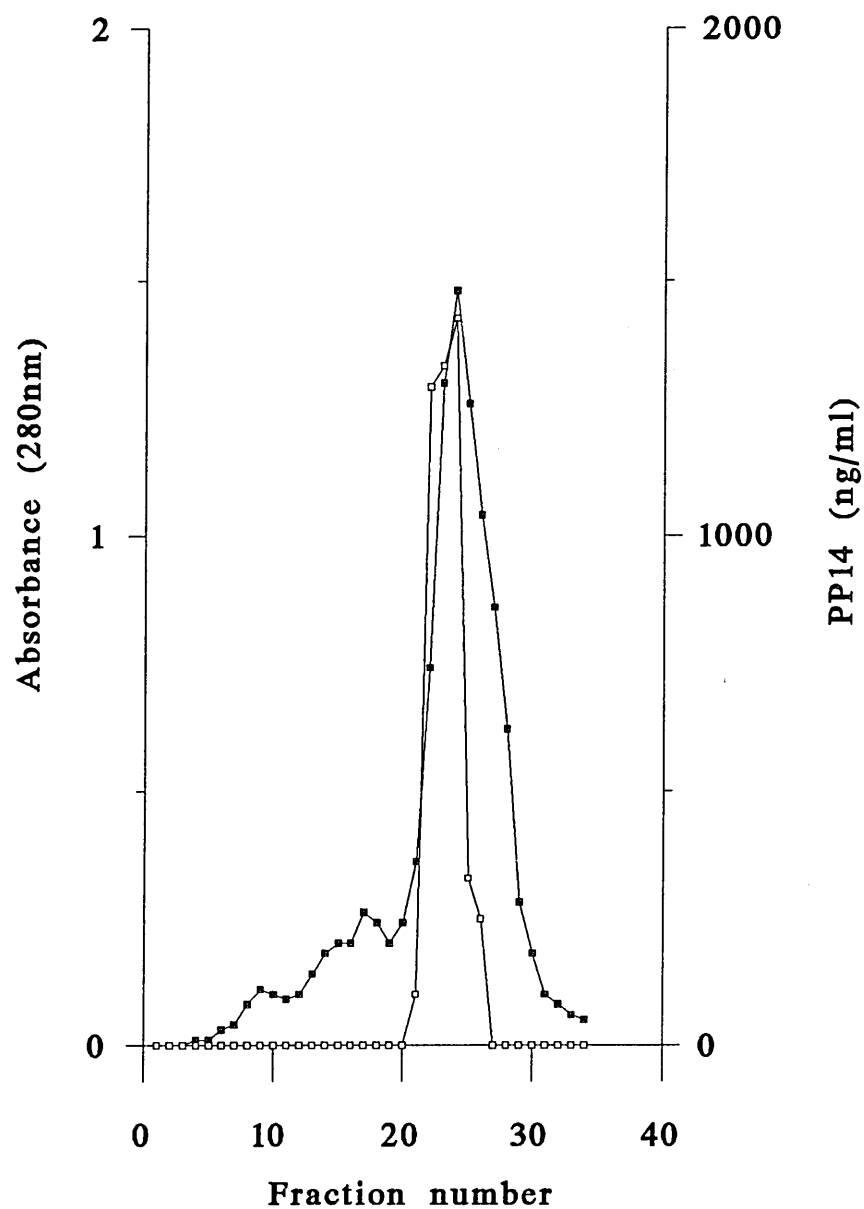
2500mls of decidual extract was loaded onto the ion exchange DEAE Fast Flow Sepharose column. Contaminating haemoglobin passed through the column and after washing the column, PP14 was eluted in one step with 0.25M NaCl. The volume of the eluent was 500ml and the total PP14 content 190 mg. After this step the degree of purity was 6% (Table 2.2).

500mls of eluent was mixed with 250mls of 4M ammonium sulphate, centrifuged for 30 minutes at 4°C at 10,000g, then the supernatant loaded onto the hydrophobic interaction hexyl agarose column (Figure 2.1). There was no PP14 detected by radioimmunoassay in the pellet obtained from the centrifugation. All the protein loaded bound to this column. An ammonium sulphate gradient was applied and the protein bound was eluted as a broad peak. PP14 was measured as a distinct peak within this broader peak. The volume of the fractions containing PP14 was 70ml and the purity after this step was 22% (Table 2.2).

After concentrating the pooled fractions they were loaded onto a gel filtration Superdex 200 HR column in 5 aliquots each of 5mls (Figure 2.2). PP14 was eluted in fractions 21-25 at 210-235 mls.



**Figure 2.2:** The trace obtained from the hydrophobic interaction chromatography step. 10ml fractions were collected, the absorbance is indicated by the closed squares and the PP14 concentration measured in each fraction by radioimmunoassay is indicated by the open squares. The dotted line indicates the salt concentration.



**Figure 2.3:** The trace obtained from the gel filtration chromatography step. 5ml fractions were collected, the void volume was 110mls. The absorbance is indicated by the closed squares and the PP14 concentration measured in each fraction by radioimmunoassay is indicated by the open squares.

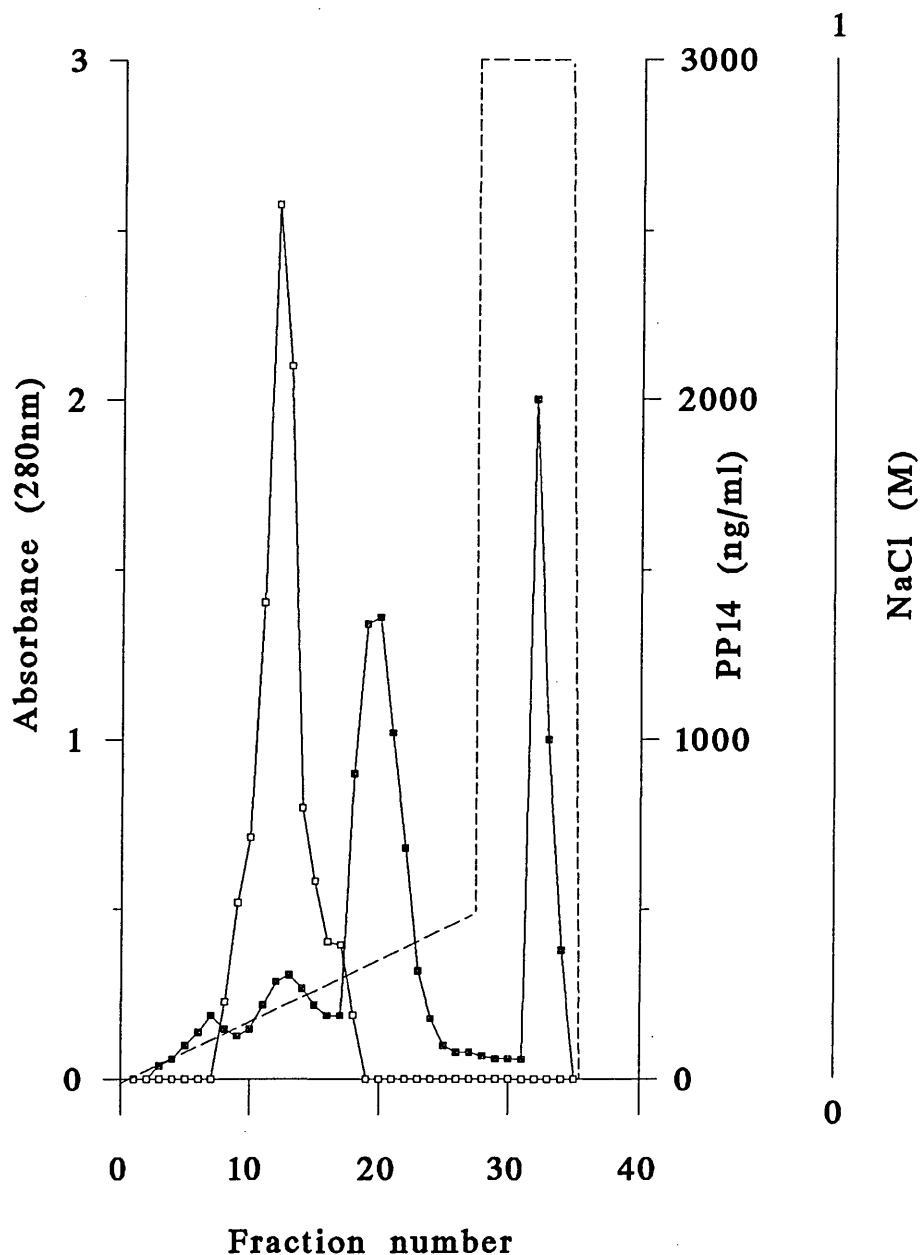
The fractions containing PP14 were loaded onto a ion exchange Hiload Q Sepharose column (Figure 2.3). PP14 was eluted with a salt gradient at 0.15M. The PP14 peak was distinct from the other peaks obtained, in particular the albumin peak.

The results of the SDS-PAGE analysis of the steps of the method are shown in Figure 2.4. The band of albumin appears to have migrated slightly faster in the lanes containing protein at a later stage of purification, the reason for this is not known.

The results, recovery and degree of purity obtained from each step in the purification are summarised in Table 2.2. The final pool of PP14 had a volume of 50mls containing 76mg of PP14 with 97% purity.

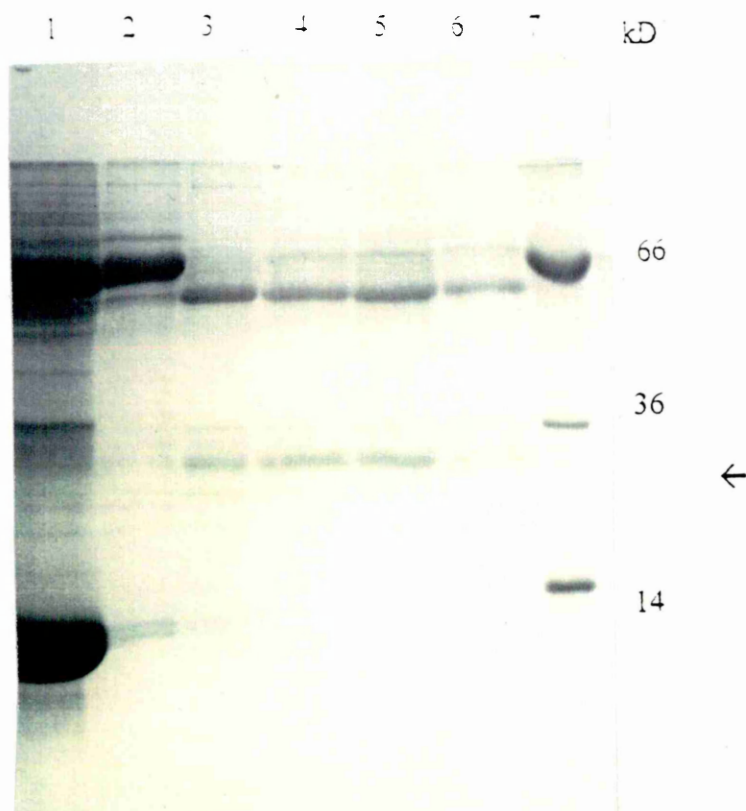
### **2.3.2 SDS-PAGE and Western blotting**

The results of the SDS-PAGE and Western blots are shown in Figure 2.5. The PP14 obtained was 97% pure by total protein estimation and ran as a single band on SDS-PAGE gels at 28,000 Da. The protein was immunoreactive with polyclonal and monoclonal antibodies previously shown to be reactive for PP14 (*Bolton et al., 1983; 1987*)



**Figure 2.4:** The trace obtained from the ion exchange chromatography step. 10ml fractions were collected. The absorbance is indicated by the closed squares and the PP14 concentration measured in each fraction by radioimmunoassay is indicated by the open squares. The dotted line indicates the salt concentration.





**Figure 2.5:** SDS-PAGE analysis of the steps of the purification. The molecular weight markers are listed on the right. The band of PP14 is arrowed.

Lane 1: Crude decidual extract

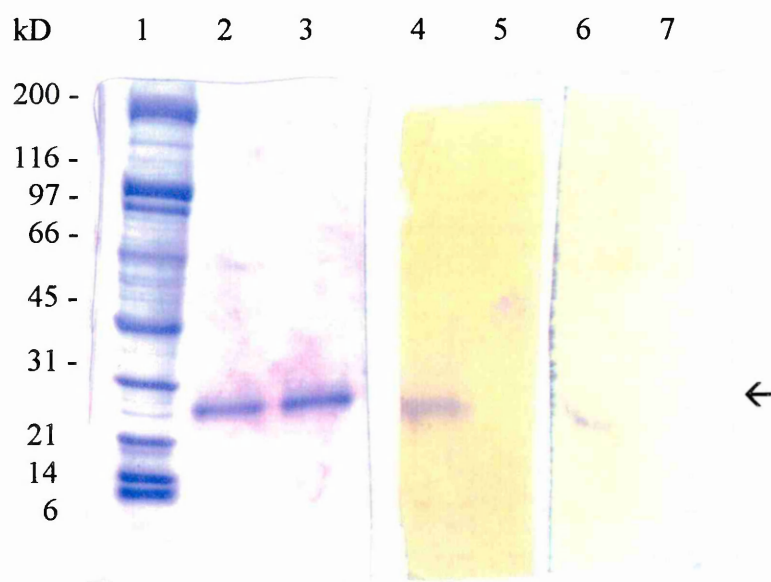
Lane 2: Sample after the first step, ion exchange

Lane 3, 4, 5, 6 : Samples after the second and third steps, hydrophobic interaction and gel filtration, fractions from the gel filtration column.

Lane 7: Molecular weight markers

Purification Step	Volume (ml)	Total protein (mg/ml)	PP14 (ug/ml)	Total PP14 (mg)	Recovery of PP14 (%)	Purity (%)
Decidual Extract	2500	16	80	200	100	0.5
Ion exchange eluent	500	6.8	380	190	95	6
Hydrophobic interaction eluent	70	10.8	2357	165	82.5	22
Gel Filtration eluent (5 runs)	125	1.8	920	115	57.5	51
Ion exchange eluent	50	1.6	1518	76	38	97

**Table 2.2:** Details of the total protein content, PP14 content, recovery and purity of PP14 at each step in the purification procedure.



**Figure 2.6:** SDS-PAGE gels and Western blots of purified PP14. The molecular weight markers are listed on the left. The band of PP14 is arrowed.

Lane 1: Molecular weight markers (kD)

Lane 2: PP14

Lane 3: PP14

Lane 4: Western blot of PP14 probed with polyclonal antibody to PP14

Lane 5: Control blot of molecular weight markers probed with polyclonal antibody to PP14

Lane 6: Western blot of PP14 probed with monoclonal antibodies to PP14

Lane 7: Control blot of molecular weight markers probed with monoclonal antibody to PP14

## 2.4 DISCUSSION

Several methods have been published detailing the purification of PP14 (*Westwood et al., 1988; Bell et al., 1987; Riittinen et al., 1991*). However these methods either have yields of a few  $\mu\text{g}$  of PP14 or use harsh or unsuitable reagents in the method.

The method described here overcomes these problems to some degree. Although this method has several steps, it is possible to complete an entire run from tissue to purified protein in 3 days, including the overnight dialysis step at the beginning of the method. The method was designed to enable the steps to follow on from each other without interrupting the method to assay fractions or dialyse the sample. The rationale behind the use of DEAE column at the beginning of the method was that it would remove haemoglobin, clean up the sample and concentrate it. To simplify the identification of PP14, protein bound to this column was eluted in one step, eliminating the need to assay fractions for PP14. Subsequent steps were relied on for further resolution of the bound proteins.

The hexyl agarose column requires a high salt concentration for proteins to bind to it, and so it was natural to place this step after the ion-exchange step which leaves the protein in a high salt buffer. This step was not sufficiently reproducible to enable the prediction of the eluted fractions containing PP14, and a radioimmunoassay was routinely set up after this step to determine which fractions to pool for the gel filtration step. This step removed much of the albumin in the sample.

The gel filtration step was an essential part of the purification to remove high and low molecular weight contaminants.

The final ion exchange column separated PP14 from other remaining proteins, resulting in a pure sample that ran as one band on SDS-PAGE.

Results from this study demonstrate a method capable of purifying 75mg of active PP14 in one run with a final purity of 97%. This provided ample PP14 for activity investigations. The purity of the protein was confirmed independently using an HPLC column and acetonitrile elution (*SJ Yewdall, personal communication*).

## **2.5 CONCLUSION**

PP14 was purified in high yield and purity using a combination of chromatography techniques. On SDS-PAGE gels the protein ran at 28,000 Da, the published molecular weight of PP14. The isolated protein was demonstrated to be immunologically identical to PP14 by the binding of characterised antibodies to a Western blot of the purified protein.

# CHAPTER THREE: ACTIVITY STUDIES

## 3.1 INTRODUCTION

### 3.1.1 Immunosuppressive activity of PP14

Previous studies on PP14 have established that it has immunosuppressive activity. This activity was initially observed in mixed lymphocyte reactions (MLRs) (*Bolton et al., 1987*) and mitogen-stimulation assays (*Pockley & Bolton, 1990*) where the immunosuppressive activity of crude decidual extract could be removed with an immunoadsorbent gel coupled to monoclonal antibodies against PP14.

In immune responses the activation of lymphocytes and accessory cells such as monocyte-macrophages is increased by a process of mutual stimulation. Lymphocytes respond to mitogen or antigen stimulation by releasing cytokines including interleukin-2 (IL-2), interferon- $\gamma$  ( $\gamma$ -IFN) and tumour necrosis factor (TNF). These cytokines, in particular IL-2, activate lymphocytes and stimulate their proliferation. TNF and  $\gamma$ -IFN also activate monocyte-macrophages which in turn release interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF. These cytokines act on the lymphocytes causing further activation and a positive feedback loop is created. It has been suggested that the immunosuppressive activity of PP14 observed against lymphocytes may be mediated through the suppression of IL-1 release from monocyte-macrophages (*Pockley & Bolton, 1989*). This would create a break in the positive feedback cycle, and limit the continued activation of lymphocytes.

In the uterine environment the presence of activated NK-like decidual large granular lymphocytes and activated macrophages is a potential risk to the fetus. The release of IL-2, in particular, would be detrimental to fetal tissue because IL-2 is a crucial molecule in the activation response. Secretion of IL-2 activates lymphocytes to release  $\gamma$ IFN, which stimulates the expression of MHC antigens. Class I antigens are present on some

trophoblast cells and their expression may lead to recognition and rejection by the maternal leukocytes. PP14 is proposed as one of the molecules that keeps the immunological environment of the decidual tissue in balance by preventing the release of IL-2.

Previous studies established that PP14 suppresses the proliferation of peripheral blood mononuclear cells in response to mitogen stimulation and in MLRs. It also has been shown to suppress the release of IL-2 in mitogen stimulation experiments.

In the study described here this work was extended to investigate the ability of PP14 to affect PBMCs directly stimulated by IL-2. This may be more relevant to the situation in decidual tissue. Although mitogen-stimulation of the decidual leukocytes may occur, it is unlikely to be a factor in the process of normal pregnancy. Similarly mixed lymphocyte reactions as a model for materno-fetal interactions may not accurately parallel the physiological situation. However the lymphocytes found in the decidua are activated and could be expected to release IL-2, the fact that this does not appear to lead to immune activation in normal pregnancy could indicate a role for PP14 in suppression of the response of these cells to IL-2. Therefore it is important to establish if PP14 can suppress the direct effect of IL-2.

### **3.1.2 U937 cell line**

The growth of the cell line U937 is IL1 dependent. This cell line is from a patient with histocytic lymphoma (*Sundstrom & Nilsson, 1976*) and shows some monocyte-like characteristics including the secretion of IL-1 (*Palacios et al., 1982*). This attribute made it a suitable cell line to investigate further the activity of PP14. If the theory that PP14 suppresses IL-1 secretion is true, it can be expected that the growth of this cell line would be suppressed in the presence of PP14.

If PP14 were to suppress the growth of U937 cells there would be advantages to its use as a way to study PP14 activity, and it could be used to develop a bioassay for PP14.



The previous methods of investigating the activity of PP14 have involved the isolation of peripheral blood mononuclear cells. The use of these methods immediately introduces variations into the study. There is a natural variation between the responses of the cells from different donors and variations are also found in the responses from day to day with the same donor. The preparation of the cells afresh for each experiment introduces further opportunities for day to day differences. The use of a cell line as the source of cells for the experiments to study the activity of PP14 removes many of these variables and should provide a more reproducible response.

An additional consideration is the time consuming nature of lymphoproliferation assays. The doubling time of U937 is less than 48 hours. Unlike primary cell cultures which take several days to undergo stimulation and then enter a phase of growth, cell lines can be maintained in a constant logarithmic growth phase. Thus assays using cell lines can be carried out with shorter incubation times since the cells are already growing strongly, and without the preliminary separation procedures involved in primary cell culture.

### **3.1.3 Aims of the study**

There were three aims to this part of the study:

1. To investigate the effect of PP14 on IL-2 stimulated PBMCs.
2. To investigate the effect of PP14 on U937 cells.
3. To develop a bioassay for PP14 based on the U937 cell line.

## **3.2 MATERIALS AND METHODS**

The reagents and equipment used are listed in Appendix I. All assays were carried out in triplicate and all procedures were carried out using aseptic technique.

### **3.2.1 Isolation of peripheral blood mononuclear cells from whole blood.**

Venous blood was collected from healthy volunteers into vacutainers containing lithium heparin as an anticoagulant. After gentle mixing the blood was diluted 1:1 with RPMI medium and separated as described by Boyum (1968) by layering 20mls of blood onto 10mls of lymphopaque separation medium then centrifuging at 400g for 20 minutes. The layer containing the PBMC was carefully removed and washed by centrifugation 3 times with RPMI. Finally the cells were resuspended in 1ml of AIM V medium and the cell numbers and viability assessed by Trypan Blue dye exclusion. Equal volumes (20 $\mu$ l) of cell suspension and 0.2% Trypan Blue in PBS were mixed and a portion of the sample counted using a haemocytometer. The cells were resuspended at a concentration of  $1 \times 10^6$ /ml in AIM V medium.

### **3.2.2 Stimulation of peripheral blood mononuclear cells with interleukin-2**

PBMCs were isolated as described and cells suspended in 100 $\mu$ l aliquots of AIM V medium were placed into each of the central 60 wells of 96 well plates ( $1 \times 10^5$  cells/well). The surrounding wells were filled with culture medium to prevent evaporation from the plate. The cells were cultured with 50 $\mu$ l of interleukin-2 (IL-2) diluted in AIM V at concentrations of 0.5-500U/ml. The volume in each well was made up to 250 $\mu$ l with 50 $\mu$ l of PBS and 50 $\mu$ l of AIM V. Control wells contained AIM V instead of IL-2. The cells were incubated at 37°C in 5% CO<sub>2</sub> and 100% humidity for 24, 48, 72 and 96 hours. 24 hours before the end of the incubation time 25 $\mu$ l of AIM V medium containing 1 $\mu$ Ci of <sup>3</sup>H-Thymidine was added to each well. At the end of the experiment the cells were harvested using a semi-automatic cell harvester. The cell harvester aspirates the cells onto filter mats and rinses the wells with 0.9% NaCl to ensure all the cells are removed. The filters were left to dry then were transferred into

scintillation vials, 2mls of liquid scintillation fluid was added to each tube and after thorough mixing the samples were counted in a liquid scintillation counter for 10 minutes.

### **3.2.3 Addition of PP14 to IL-2 stimulated PBMCs**

PBMCs were isolated and cultured as described in the previous section, but this time with the addition of PP14 to the cells. PP14 purified using the method in chapter 2 and diluted in PBS was added to the cells to achieve final concentrations in the range 12.5-800µg/ml. The PP14 samples were added in 50µl, replacing the 50µl of PBS added in the previous experiment except for the control wells which contained PBS instead of PP14. The cells were cultured and harvested as in the previous experiment.

### **3.2.4 Maintenance of U937 cells**

U937 cells were maintained in continuous culture in RPMI medium supplemented with 10% foetal calf serum, 200U/ml penicillin 200µg/ml streptomycin and 2mM glutamine (RPMI+). The cells were maintained as a stock culture in 75cm<sup>2</sup> flasks and were subcultured twice a week at a split density of 1:10, with additional flasks seeded as necessary to grow up larger numbers of cells. This regime maintained the cells at a density of between  $5 \times 10^4$ /ml and  $5 \times 10^5$ /ml and ensured that the cells remained in the log phase of growth.

### **3.2.5 Culture of U937 cells**

U937 cells were removed from the 75cm<sup>2</sup> flasks, centrifuged for 5 minutes at 100g at room temperature then resuspended in fresh RPMI+ medium at the cell concentrations indicated in the figures. Cells suspended in 100µl aliquots of RPMI+ medium were placed into each of the central wells of a 96 well plate as described for the PBMC assays. 50µl of PBS was added to each well. Finally 1µCi of <sup>3</sup>H-Thymidine in 100µl of RPMI+ was added to each well to make a final volume of 250µl. The cells were cultured overnight at 37°C in 5% CO<sub>2</sub> and 100% humidity and harvested and counted as described for the PBMC experiments.

### **3.2.6 Addition of PP14 to U937 cells**

U937 cells were cultured as described in the previous section with the addition of PP14. 50µl of PP14 sample diluted in PBS was added to each well at concentration in the range 1.5-200µg/ml, PBS was added to the control wells.

### **3.2.7 Immunoabsorption of PP14**

Controls for the experiments described were the removal of PP14 from the samples by immunoabsorption. The samples before and after treatment were added to U937 cells as described in the previous section. Monoclonal antibodies to PP14 were generated as detailed in Appendix III and linked to cyanogen-bromide activated Sepharose 4B gel. The difference in activity between absorbed and unabsorbed samples was analysed statistically using Student's *t*-test. The level of statistical significance was chosen at  $P < 0.05$ .

#### **3.2.7.a Preparation of immunoadsorbent**

Monoclonal antibodies were dialysed against coupling buffer (Sodium carbonate, pH 8.3, 0.5M NaCl) and diluted to a concentration of 1mg/ml. Cyanogen bromide activated Sepharose 4B gel was equilibrated in 1mM HCl then washed with 250mls of 1mM HCl for each ml of gel. The gel was then washed quickly with coupling buffer and mixed with the dialysed antibody solution for 2 hours end-over-end at room temperature, 2.5mg of antibodies were mixed with each ml of gel. Any remaining reactive molecules on the activated gel not bound to antibodies were blocked with 0.2M glycine by end-over-end mixing for 2 hours at room temperature. The gel was then washed alternately with coupling buffer and sodium acetate buffer (0.1M, pH 4.0, 0.5M NaCl), by resuspending the gel in 30mls of buffer then centrifuging it for 5 minutes at 2000g at room temperature. This washing cycle was repeated four times. Finally the gel was washed 5 times with PBS, and stored in PBS containing 0.1% NaN<sub>3</sub> until required. Before use, the gel was rewashed with PBS 5 times to remove the preservative.

### **3.2.7.b Treatment of samples**

Samples containing up to 1 mg/ml PP14 were immunoadsorbed by mixing with an equal volume of immunoadsorbent gel on an end-over-end mixer for two hours at 4°C.

### **3.2.7.c Effectiveness of immunoabsorbent**

The effectiveness of the immunoabsorption was checked by running the sample before and after on SDS-PAGE gels. Control samples were incubated with Sepharose 4B blocked with glycine only. This was to demonstrate the specificity of the immunoabsorbent as well as the successful removal of PP14. Therefore, a sample with some minor contaminating bands was chosen to demonstrate these bands did not bind to the gel. The samples were also assayed using the radioimmunoassay to confirm the removal of PP14.

### **3.2.8 Viability of U937 cells**

To confirm that PP14 did not have a cytotoxic effect on the U937 cells parallel experiments were set up with PP14 added to the cells at the highest concentrations. Instead of harvesting the cells, at the end of the incubation period the cells in each well were resuspended using a 1ml pipette, a portion was mixed with an equal volume of Trypan blue and a viable cell count carried out microscopically using a haemocytometer.

### **3.2.9 U937 bioassay for PP14**

The U937 cell line was used to develop a bioassay for PP14. Samples were incubated with cells as described in the previous sections but instead of adding the samples at specific concentrations they were incubated with the cells at a fixed range of sample dilutions. The results were plotted as titration curves and used to express the activity of the PP14 samples in Units/ml as described in the results section. 2 samples were analysed 4 times each to establish the inter- and intra-assay variation, and the assay detection limit.

### **3.2.10 Effect of temperature on the activity of PP14**

To test the temperature stability of the activity a sample of PP14 was divided into 4 portions. These were treated as follows:

1. 4°C for 30 minutes
2. 20°C for 30 minutes
3. 37°C for 30 minutes
4. Boiled for 5 minutes at 100°C

### **3.2.11 Effect of pH on the activity of PP14**

A sample of PP14 diluted in PBS was split into 4 portions. High and low pH buffers were made up (0.5M Tris, pH 11.0, 0.5M sodium acetate, pH 2.5) and added to the samples. It had previously been calculated how much of each buffer needed to be added to the PP14 samples to achieve test pH values of 3.0, 5.0 and 9.0 by adding the high and low buffers drop by drop to samples of PBS while measuring the pH. The PP14 samples were stored at the test pH values at 4°C for 30 minutes and then the pH of each sample was adjusted, with the appropriate buffer, to pH 7.2, the starting pH. The samples were then made up to a constant volume with water, and incubated with U937 cells. As additional controls neutralised buffers alone were added to U937 cells..

### **3.3 RESULTS**

#### **3.3.1 The effect of incubation time on the response of peripheral blood mononuclear cells to a range of IL-2 concentrations**

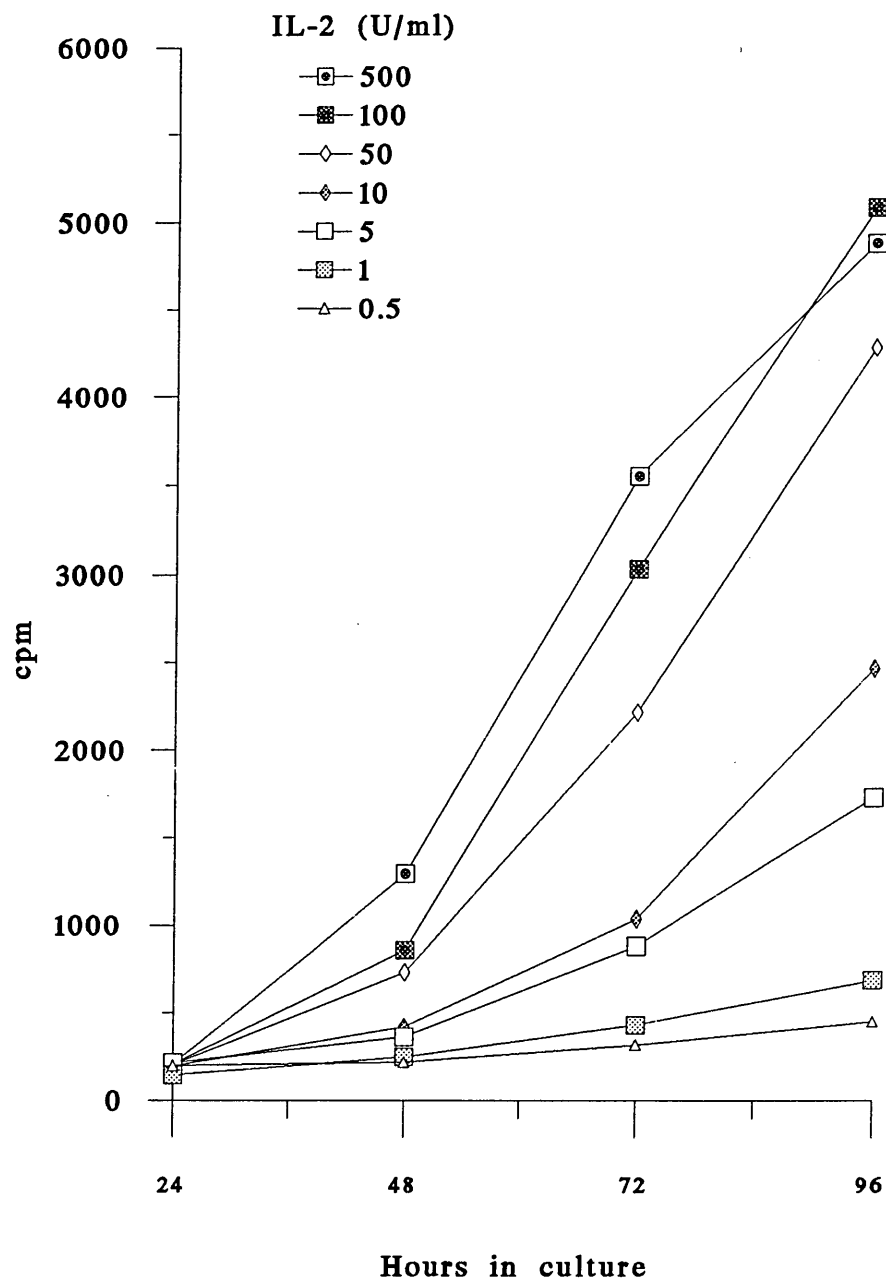
Peripheral blood mononuclear cells (PBMCs) were stimulated with IL-2 for 24, 48, 72 and 96 hours at concentrations of 500, 100, 50, 10, 5 and 1 units/ml. The results of this experiment are shown in Figure 3.1. IL-2 stimulated uptake of  $^3\text{H}$ -thymidine by the cells in a dose-dependent manner, with the extent of stimulation increasing with time. From these results 72 hours was chosen as an adequate stimulation period, with an IL-2 concentration of 100U/ml.

#### **3.3.2 The effect of PP14 on $^3\text{H}$ -Thymidine uptake by peripheral blood mononuclear cells (PBMCs) in response to IL-2**

PP14 was added at different concentrations to PBMCs stimulated with IL-2. The results are shown in Figure 3.2. There was a dose-dependent suppression of the uptake of  $^3\text{H}$ -thymidine by the cells when they were incubated with PP14 at concentrations in the range 12-800 $\mu\text{g/ml}$ . There was a wide variation in the immunosuppressive activity observed in different samples, this can be seen by the large standard error of the mean (S.E.M.) values.

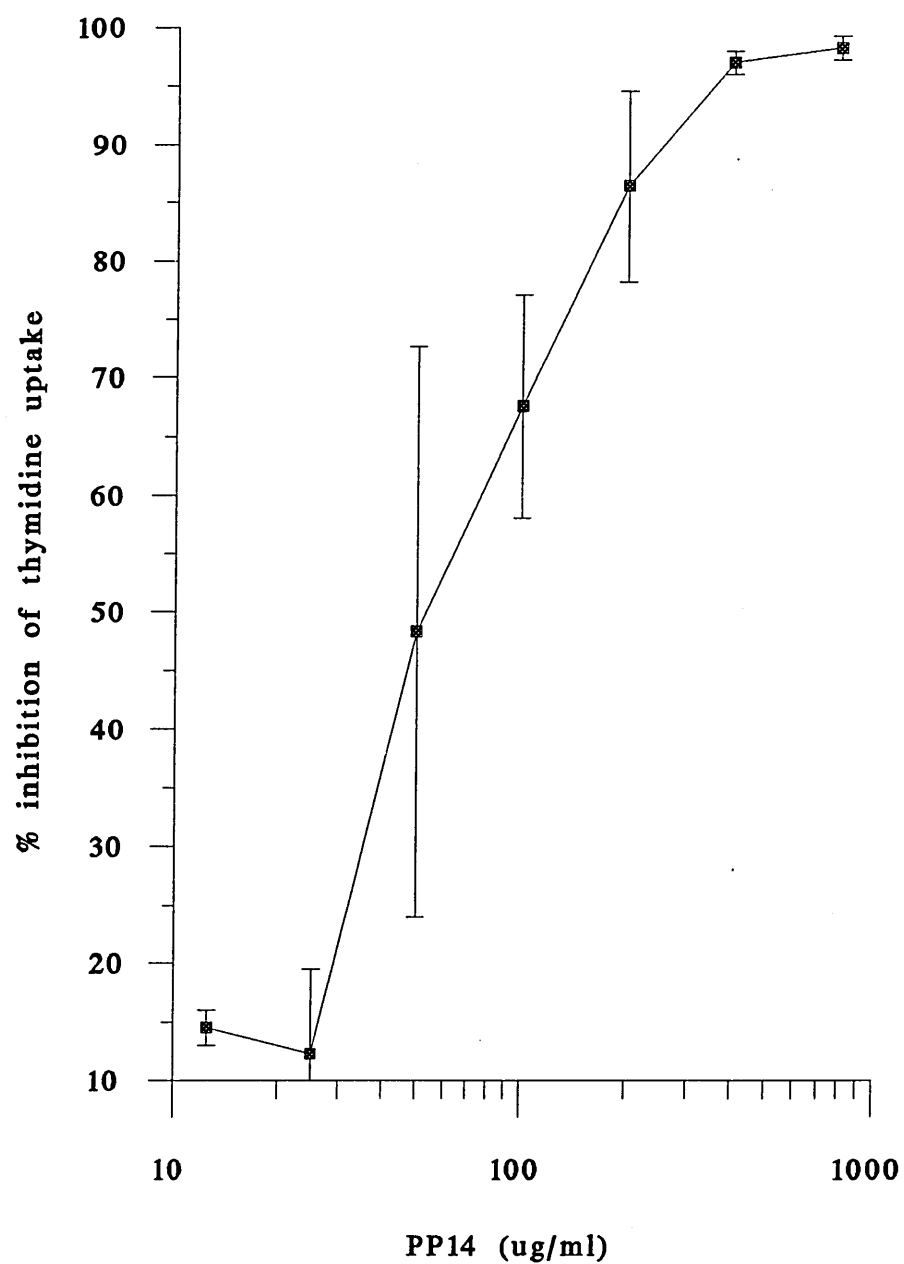
#### **3.3.3 The effect of PP14 on $^3\text{H}$ -Thymidine uptake by U937 cells**

Figure 3.3 shows the suppression of the growth of the cell line U937 when incubated with PP14 at the concentrations indicated. There is a dose-dependent response between the concentration of PP14 and the suppression in the range 1.5-100 $\mu\text{g/ml}$  with a slight drop in suppressive activity at the highest concentration of 200 $\mu\text{g/ml}$ . As with the results seen with IL-2 there is wide variation seen between PP14 samples at the same concentration.

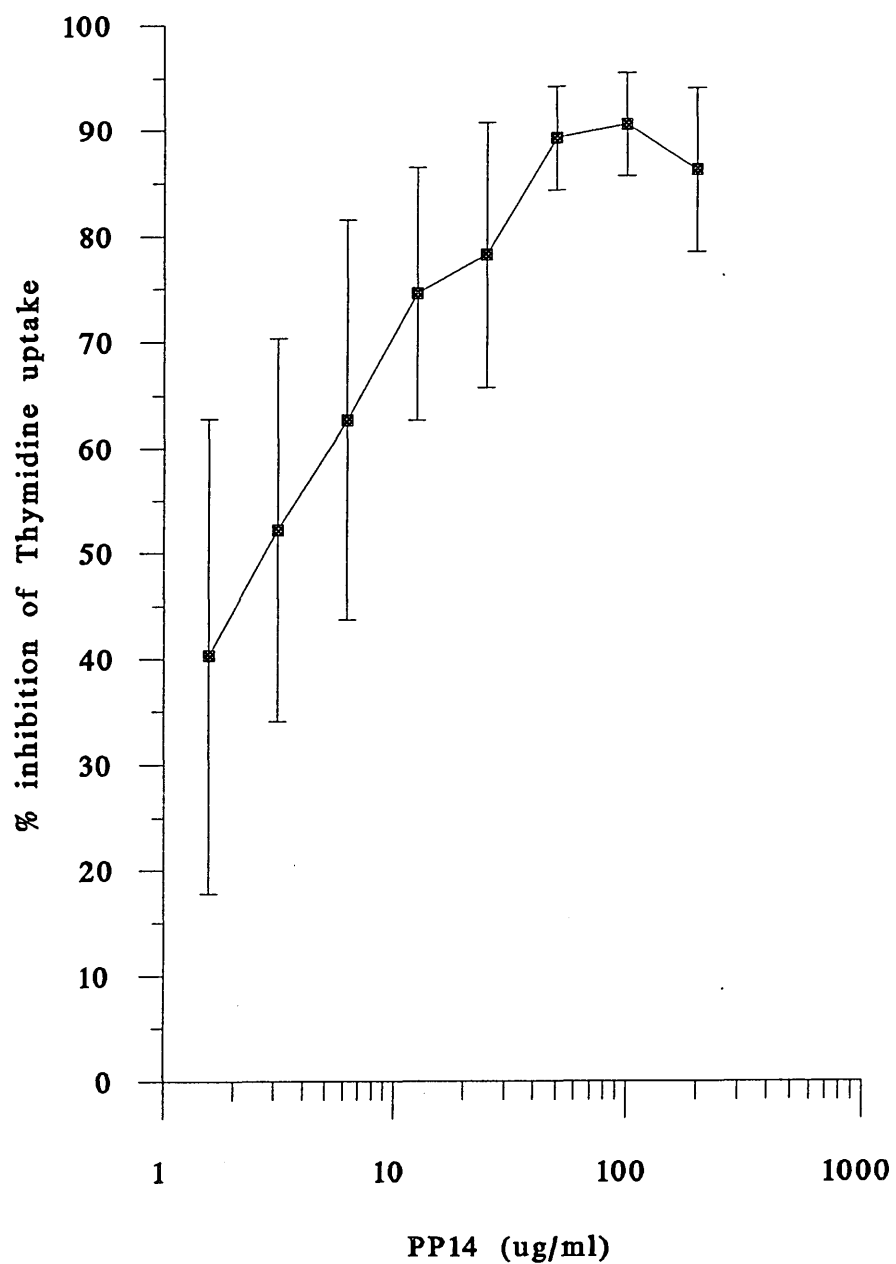


**Figure 3.1:** Time course of the uptake of <sup>3</sup>H-thymidine by peripheral blood mononuclear cells stimulated by IL-2 at concentrations in the range 0.5-500U/ml.





**Figure 3.2:** The effect of PP14 concentration on the uptake of  $^3\text{H}$ -thymidine by PBMC stimulated with IL-2. (n=3). Results are expressed as means  $\pm$  S.E.M.



**Figure 3.3:** The effect of PP14 concentration on the uptake of <sup>3</sup>H-thymidine by the cell line U937 (n=6). Results are expressed as means  $\pm$  S.E.M.

### **3.3.4 Viability of U937 cells**

The viability of U937 cells with or without PP14 was over 95%. Therefore the suppression of the uptake of  $^3\text{H}$ -Thymidine by U937 cells was not due to a cytotoxic effect of PP14.

### **3.3.5 The effect of cell number on the suppression of the growth of the cell line U937 by PP14**

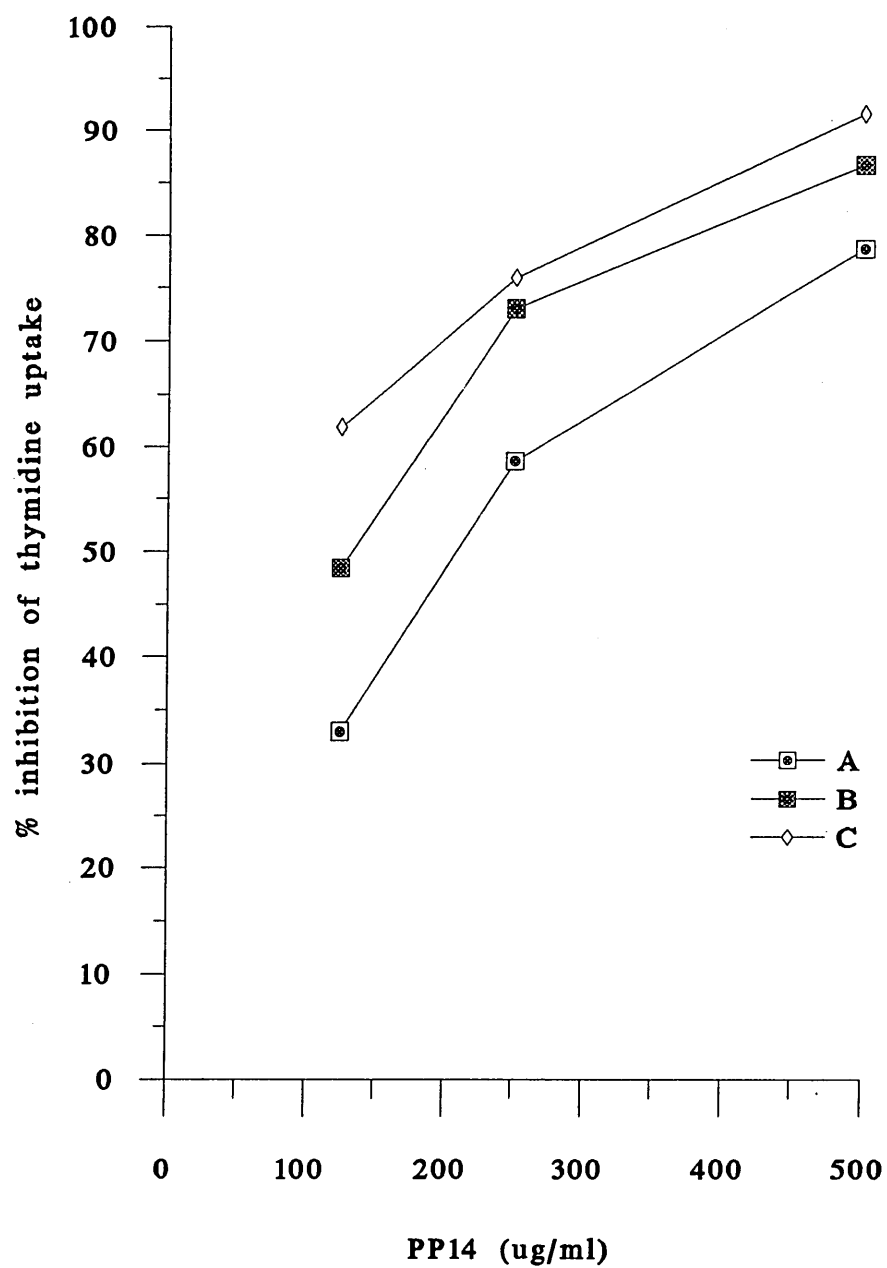
U937 cells were incubated at three different cell concentrations with the same sample of PP14 to establish the effect of cell concentration on the extent of growth suppression. The results are shown in Figure 3.4 and show that the pattern of dose-dependent suppression is independent of the cell concentration. The results from this experiment were used to determine a cell concentration for later experiments. One of the purposes of investigating the U937 cell line as a basis for an assay was to develop a rapid method of measuring the activity of PP14. The  $^3\text{H}$ -Thymidine counts obtained with a cell concentration of  $1 \times 10^6/\text{ml}$  were approximately 10 x higher than with  $1 \times 10^5/\text{ml}$  in proportion to the number of cells. Therefore the samples with more cells could be counted for a shorter time since 10,000 counts should be accumulated to minimise the radioactive counting error. A cell concentration of  $1 \times 10^6/\text{ml}$  was chosen for subsequent experiments.

### **3.3.6 Removal of activity by immunoabsorbent gel against PP14**

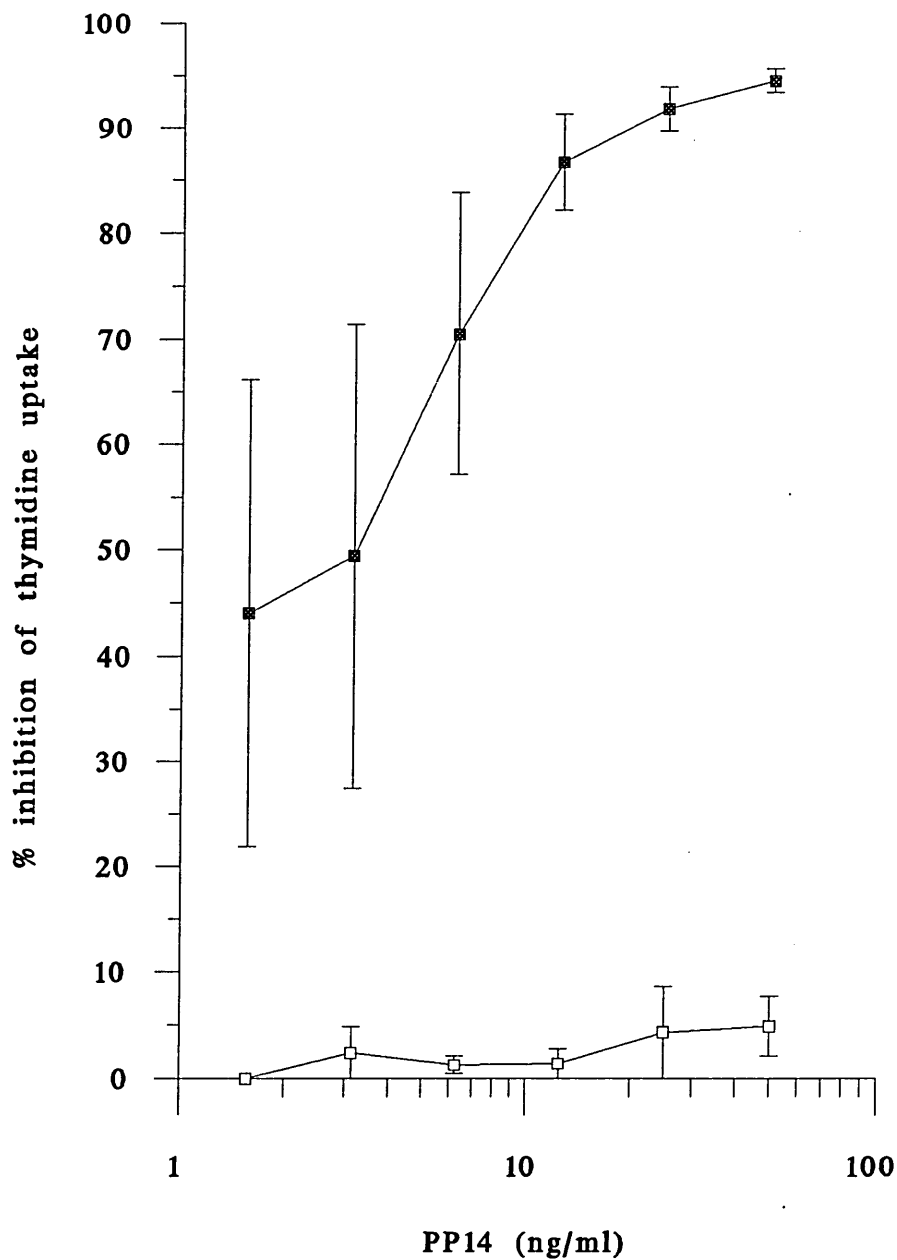
After treatment of the PP14 samples with the immunoabsorbent gel the activity against U937 dropped as shown in Figure 3.5. The removal of activity was significant at all concentrations ( $50\text{-}12.5\mu\text{g}/\text{ml}$ ,  $P<0.0005$ ;  $6.25\mu\text{g}/\text{ml}$ ,  $P<0.001$ ;  $3.1\mu\text{g}/\text{ml}$ ,  $P<0.05$ ).

### **3.3.7 SDS-PAGE analysis of the effectiveness of immunoabsorption with monoclonal antibody gel**

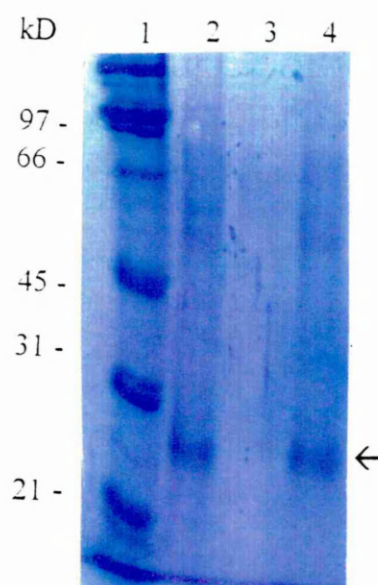
Figure 3.6 shows the SDS-PAGE gel of the samples before and after treatment with the immunoabsorptive gel. In addition, the control gel blocked with glycine only was mixed



**Figure 3.4:** U937 cells cultured overnight at three different concentrations of cell/ml with the same sample of PP14. A:  $1 \times 10^6$ /ml, B:  $5 \times 10^5$ /ml, C:  $1 \times 10^5$ /ml.



**Figure 3.5:** The effect of treating PP14 samples with a monoclonal antibody immunoabsorbent gel on its ability to inhibit the uptake of  $^3\text{H}$ -thymidine by U937 cells. The results are expressed as means  $\pm$  S.E.M. (n=4) Closed squares are unabsorbed, open squares are immunoabsorbed.



**Figure 3.6:** SDS-PAGE analysis of PP14 sample before and after incubation with monoclonal antibody-substituted and glycine blocked Sepharose gel. The band of PP14 is arrowed.

Lane 1: Molecular weight markers

Lane 2: Sample before incubation with immunoabsorbent

Lane 3: Sample after incubation with monoclonal antibody Sepharose gel

Lane 4: Sample after incubation with control Sepharose gel

with a portion of the sample and also run on the gel. The results demonstrate the effectiveness of the monoclonal antibody gel in specifically removing PP14 from the sample and show that this is not a non-specific effect since the Sepharose blocked with glycine does not bind PP14 (Figure 3.6).

### **3.3.8 Analysis of PP14 samples measured in units of activity**

In view of the wide variation observed between different samples of PP14 it was decided to develop a method of expressing the immunosuppressive activity of PP14 in terms of units of activity instead of concentrations. Samples of PP14 were incubated with U937 cells at a range of PP14 dilutions as shown for 10 representative samples in Figure 3.7. One unit of activity was arbitrarily defined as the PP14 concentration required to suppress by 50% the uptake of  $^3\text{H}$ -Thymidine by U937 cells. This method of expressing units of activity of a growth-suppressing substance as 50% suppression has been used to define the activity of other molecules including TGF- $\beta$  (Meagher, 1991). The final dilution at which a 50% suppression would be achieved is read off the graph and gives the Units/ml of PP14 activity in that sample. The activity in all 10 of the samples shown on this graph was calculated and the results are shown in Table 3.1.

### **3.3.9 Inter- and Intra-assay variation**

The inter-assay variation was 22.5%, the intra-assay variation was 12.5%. The detection limit of the assay was defined as the level of suppression of growth that was significantly different from the controls, and was found to be 25%.

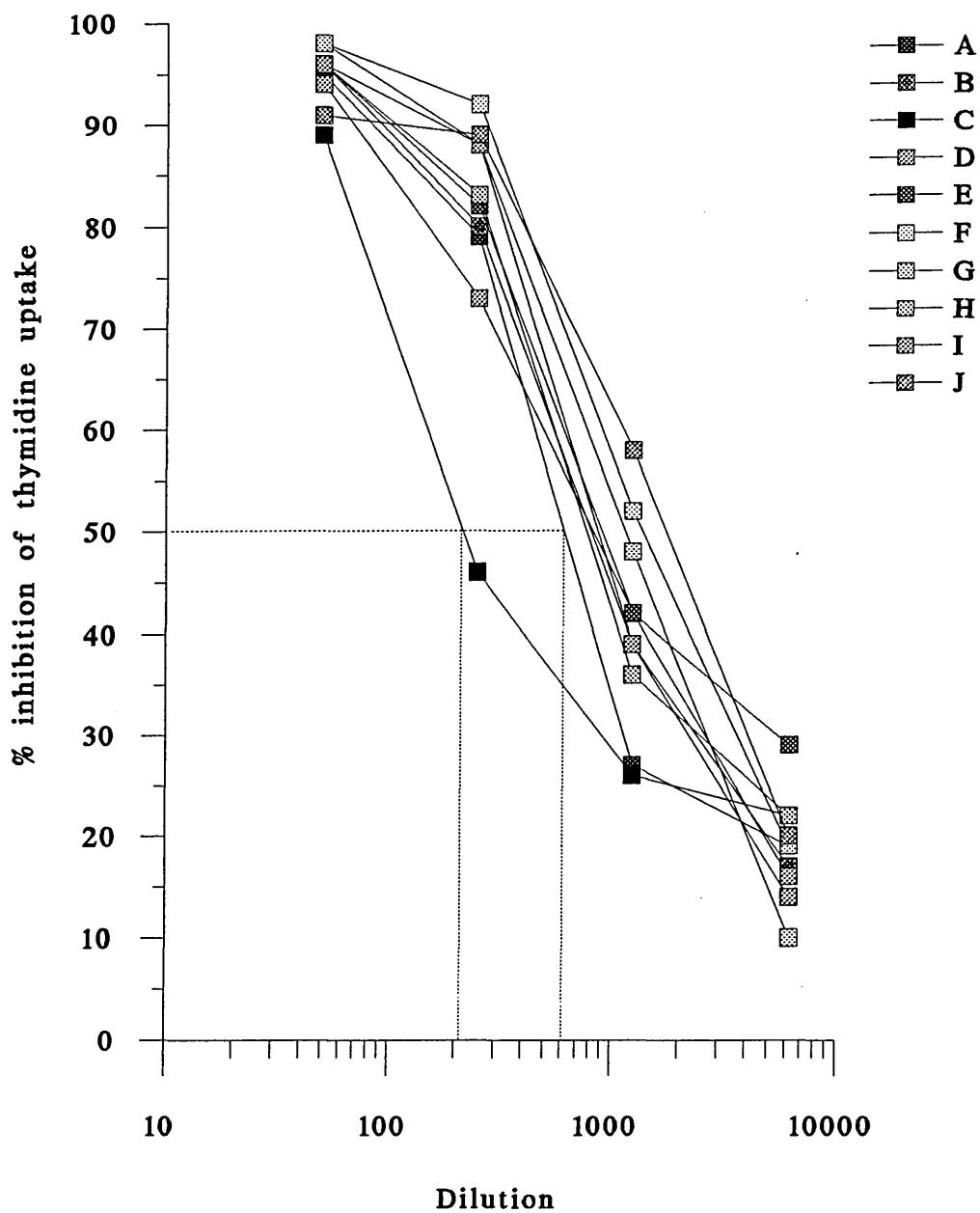
### **3.3.10 The effect of temperature on the activity of PP14**

The results of the experiment to investigate the effect of temperature on the stability of the activity of PP14 are shown in Figure 3.8. These results show that heating PP14 to 100°C destroys much of its activity. Leaving PP14 at 37°C or 20°C for 30 minutes also reduces the activity compared with an identical sample stored at 4°C for the same length of time.

### **3.3.11 The effect of pH on the activity of PP14**

The results of the exposure of PP14 to high and low pH are shown in Figure 3.9. When exposed to a pH of 3 for 30 minutes a substantial loss of PP14 activity was observed. There was also some loss of activity at pH 5 and 9.

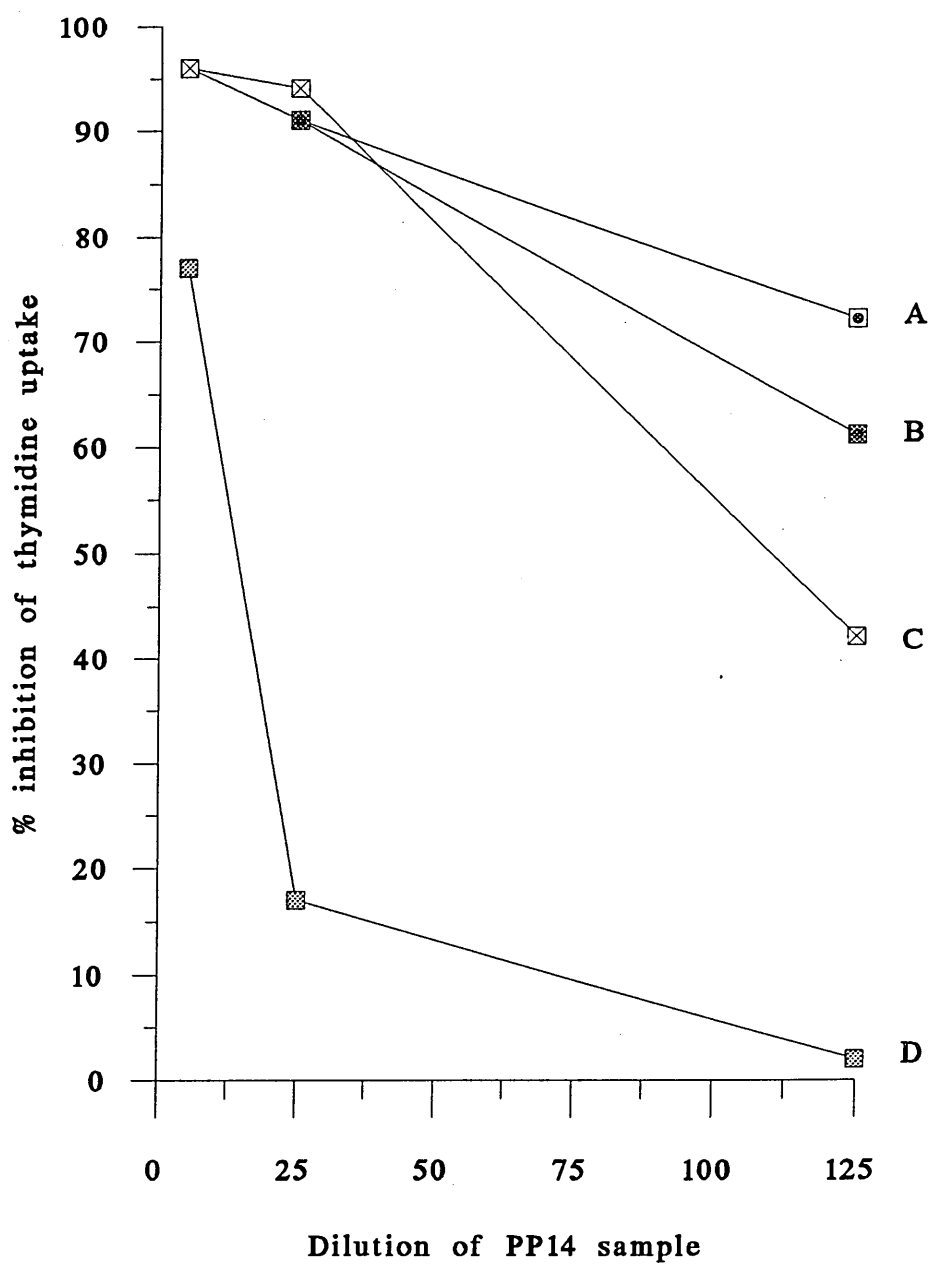




**Figure 3.7:** Inhibition of uptake of  $^3\text{H}$ -Thymidine by samples of PP14 expressed as dilutions of the original samples. The dotted lines read off the units of activity for two of the samples.

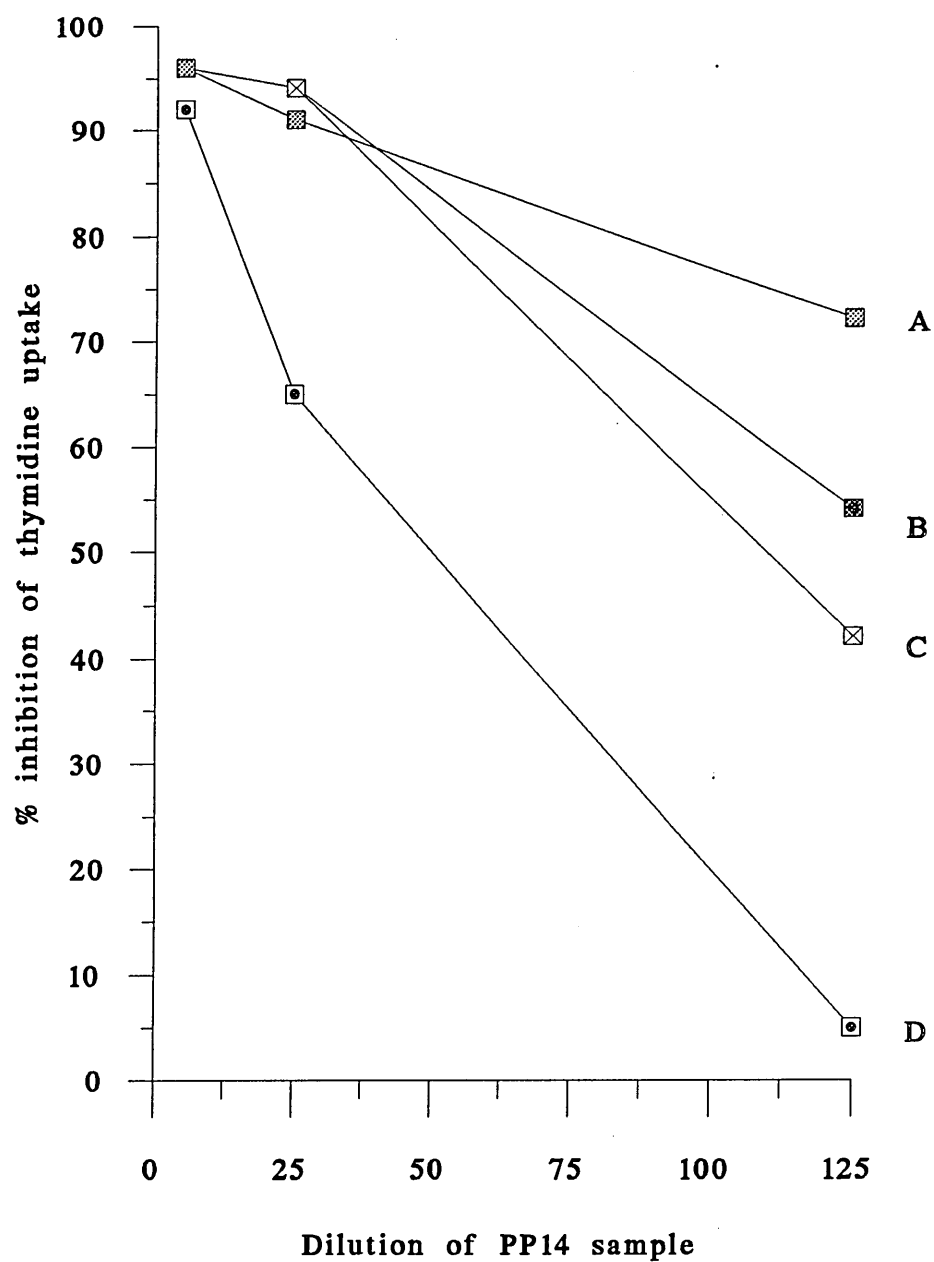
Sample	Activity (Units/ml)
A	600
B	800
C	215
D	800
E	880
F	1350
G	1100
H	760
I	850
J	1800

**Table 3.1** Analysis of 10 samples of PP14 expressed in Units of activity.



**Figure 3.8** The effect of temperature on the ability of PP14 to suppress the uptake of  $^3\text{H}$ -Thymidine by U937 cells.

A: 4°C, B: 20°C, C: 37°C (all 30 minutes incubation), D: 100°C (5 minutes).



**Figure 3.9:** The effect of pH on the ability of PP14 to inhibit the uptake of  $^3\text{H}$ -Thymidine uptake by U937 cells. A: No treatment, B: pH 9, C: pH 5, D: pH 3.

### **3.4 DISCUSSION**

#### **3.4.1 Inhibition of the response of peripheral blood mononuclear cells (PBMCs) to interleukin-2 (IL-2)**

PP14 has previously been shown to inhibit the response of PBMCs to mitogen stimulation and mixed lymphocyte reactions (MLRs) (*Pockley & Bolton, 1988; Bolton et al., 1987*). PP14 has also been shown to suppress the release of IL-2 from PBMC cultures (*Pockley et al., 1990*). To explain this activity it has been suggested that the mode of action of PP14 is via suppression of the release of interleukin-1 (IL-1). IL-1 and IL-2 are released by monocyte-macrophages and lymphocytes respectively as part of a positive feedback mechanism. Suppression of the release of either of these cytokines will lead to a down-regulation of this response. The release of IL-2 by lymphocytes acts in a positive feedback loop and stimulates them to proliferate and release further IL-2 as well as other inflammatory cytokines including IL-6 and  $\gamma$ -IFN which act on monocyte-macrophages and induce them to release IL-1 which also induces lymphocyte proliferation.

The results described here show that PP14 suppresses the response of lymphocytes to exogenous IL-2. This may be through the secondary path of suppressing IL-1 or it may be a direct effect on the lymphocytes.

The concentrations of PP14 which suppresses IL-2-induced proliferation (10-800 $\mu$ g/ml) are in the same range as the concentrations described previously as suppressing mitogen stimulated proliferation or MLRs.

#### **3.4.2 Inhibition by PP14 of $^3\text{H}$ -thymidine uptake by U937 cells**

The cell line U937 is dependent on IL-1 for its growth, therefore the observation that PP14 suppresses this growth as measured by  $^3\text{H}$ -Thymidine uptake is further evidence that PP14 may act through the suppression of IL-1 release. The removal of this activity

by the immunoabsorption of the samples with a monoclonal antibody gel specific for PP14 confirmed PP14 as the molecule responsible for the activity. The optimisation of this experiment resulted in the development of a routine assay for the measurement of the activity of PP14. The variation of specific activity between different preparations of pure PP14 has been a problem in the study of this molecule. However with the method of activity measurement based on the primary cell culture of PBMCs, the variability between different blood donors was a significant source of error. The U937 assay reduced this variability and provides a reproducible method of investigating the activity of PP14.

As an example of the type of experiments this assay could be used for, the temperature and pH stability of PP14 was investigated. The observation that, on exposure to room temperature, a loss of activity can be measured after only 30 minutes may explain much of the variability between different samples.

One of the other immunosuppressive molecules in the endometrium is TGF- $\beta$ , which is active at ng/ml concentrations, making it 1000x more potent than PP14. Despite the evidence that the immunoabsorbent gel specifically both removes PP14 and the immunosuppressive activity, a contamination of only 0.1% TGF- $\beta$  would significantly interfere with the results. However TGF- $\beta$  is acid stable, in fact it requires acid conditions for activation from its latent form (*Meagher, 1991*) and is often stored in acid conditions to preserve the biological activity. The total loss of activity observed with PP14 samples treated at pH 3 establishes that contamination of the sample with TGF- $\beta$  cannot be responsible for the immunosuppressive activity.

The use of U937 cells to assay the activity of PP14 provides future opportunities for further study of the activity, receptors and mode of action of PP14. Although the assay is not specific for PP14, it can be made so by the use of the immunoabsorption gel to prepare control samples.

### 3.5 CONCLUSION

PP14 suppresses the uptake of  $^3\text{H}$ -Thymidine by IL-2 stimulated peripheral blood mononuclear cells. As will be shown in Chapter 6 the concentrations at which PP14 is active are physiological within the local environment of the endometrium. The activated leukocytic cells found in the endometrium and decidua are sensitive to IL-2 stimulation. Therefore these results show that PP14 may play a role in suppressing the activation of leukocytic cells in the decidua.

The development of a bioassay for PP14 will simplify future studies of the activity, receptors and mode of action of this molecule.

# CHAPTER FOUR: CLONING AND OVEREXPRESSION OF RECOMBINANT PP14 IN *ESCHERICHIA COLI*

## 4.1 INTRODUCTION

The treatment of patients with human-derived proteins is only ethical and practical if a recombinant form of the protein is available. PP14 is a potential therapeutic protein in the treatment of recurrent miscarriage, infertility and inflammatory disease, but the cloning and expression of PP14 in a recombinant form is a pre-requisite for the investigation of any clinical application. This chapter describes the expression of PP14 as a recombinant protein.

### 4.1.1 Cloning strategy

Recombinant PP14 was cloned in *E. coli* by the insertion of cDNA for PP14 into the multiple cloning site of the pGEX-KG vector expression system (*Guan and Dixon, 1991*). This enabled the expression of the protein in frame with glutathione *S*-transferase (GST) as a fusion protein. This expression system allows the rapid and efficient purification of the fusion protein by affinity chromatography using glutathione agarose beads. pGEX-KG is a derivative of the pGEX-2T vector (*Smith and Johnson, 1988*) with an extra glycine-rich linker inserted next to the specific thrombin cleavage site between glutathione *S*-transferase and the cloned protein. This linker increases the accessibility of the cleavage site and so improves the efficiency of thrombin in the cleavage reaction which splits GST from the desired recombinant protein. The expression of proteins as fusion products may also increase their solubility, which is often a limiting factor in the yield of recombinant proteins.

Proteins that contain more than 2.9% arginines are often expressed at very low levels in *E. coli* since the arginine codons AGA and AGG are rarely found in prokaryotic genes but are common in eukaryotic genes. When *E. coli* encounters these codons the cells stop transcribing the DNA. Brinkmann et al., (1989) found a correlation between the



abundance of these codons and lack of protein expression in *E. coli*. If a protein had over 2.9% of AGA and AGG codons then the levels of expression were low or undetectable. PP14 has a AGA+AGG content of 3.3% (Julkunen *et al.*, 1988), and therefore there is a strong probability that the expression levels of this protein in *E. coli* may be low. This potential problem was overcome by transforming the *E. coli argU* gene encoding the rare arg t-RNA<sub>AGA</sub> into the pPP14.GEX clone using the plasmid pUBS250 (Brinkmann *et al.*, 1989) which carries the *argU* gene under the control of the *lac* promoter and is compatible with the pBR322 based vectors of which the pGEX derivatives are examples.

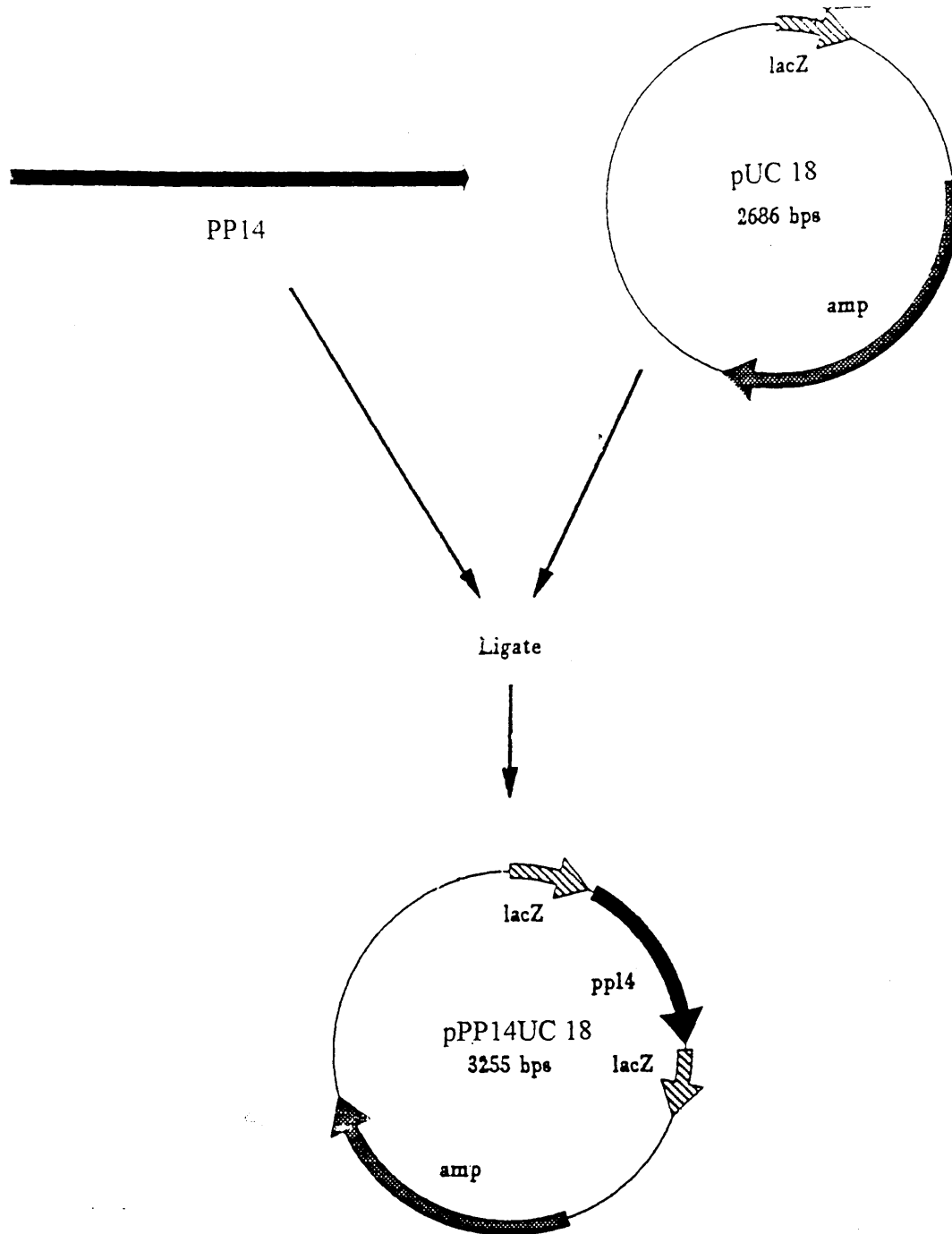
*E. coli* was chosen as the host organism for the cloning of PP14. There are some disadvantages to *E. coli*, including the lack of post-translational modifications such as glycosylation and folding which may be essential to obtain active protein. However, rapid, high level expression of recombinant proteins can be obtained in *E. coli* with low cost due to fast cell growth and cheap growth media.

cDNA coding for PP14 was prepared using the reverse transcriptase reaction after mRNA purification. The leader sequence was included in the cloning procedure since sometimes this is important for the folding of the protein. Although less relevant when the expression is carried out in *E. coli*, it leaves the option open of transferring the clone to a mammalian expression system at a later date, when this may become important. The amino acids coded for by the leader sequence can be cleaved from the expressed protein after purification, but sometimes the presence of a leader amino acid sequence makes no difference to the activity and can be ignored. In the case of PP14 the leader sequence consists of 18 amino acids, approximately 10% of the protein. The cDNA was amplified using the PCR reaction and then inserted into the cloning vector pUC 18 using blunt ended ligation. The initial use of a cloning vector facilitated the ligation subsequently into the expression vector pGEX-KG. The use of pUC 18 enabled the identification of clones containing the PP14 gene using the blue/white selection technique. This is based on the design of the position of the multiple cloning site in pUC 18, which is situated in

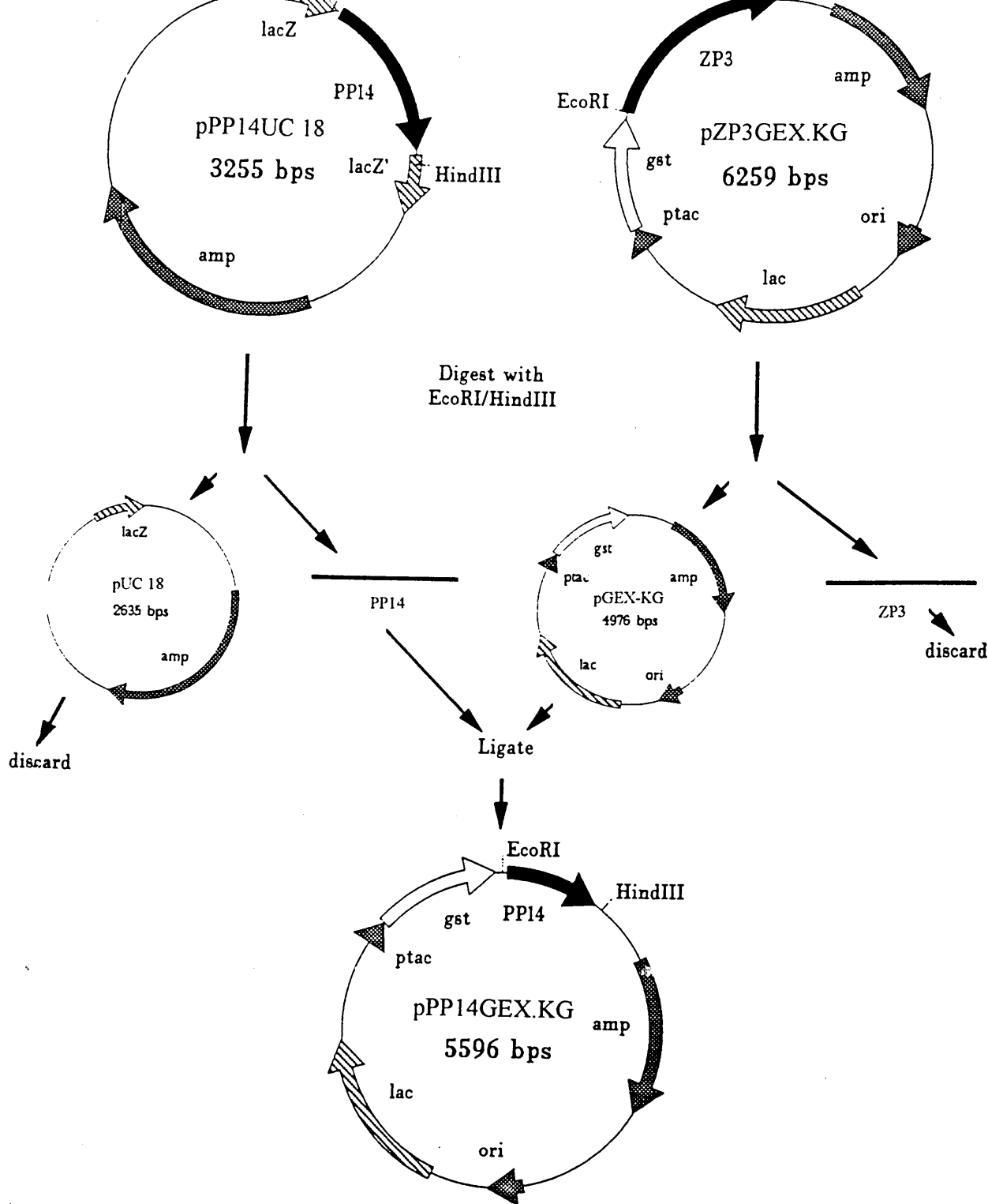
the *lacZ* gene. *LacZ* codes for  $\beta$ -galactosidase which converts 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal) blue when induced by isopropyl-thio- $\beta$ -D-galactoside (IPTG) and is inactivated when a gene is inserted into it. Colonies containing religated vector without the inserted PP14 cDNA still have the active *lacZ* gene and will be blue, while colonies containing the insert will be white. A schematic representation of this ligation is shown in Figure 4.1.

The PP14 cDNA was then religated into the expression vector pGEX-KG. The vector used was a derivative of pGEX-KG containing cDNA coding for the human zona-binding protein (hZP3) inserted into the *Eco* RI and *Hind*III sites in the multiple cloning site. Both vectors were digested with *Eco* RI and *Hind*III and the hZP3 cDNA in the pGEX-KG vector replaced with the PP14 cDNA. A schematic representation cloning strategy is shown in figure 4.2.

The *argU* gene carrying plasmid pUBS250 was then inserted into the clone. The synthesis of recombinant PP14 as a fusion protein with glutathione *S*-transferase in pGEX-KG is controlled by the *tac* promoter (*Amann et al., 1983*) which is normally repressed by the *lac* repressor located on the plasmid until induction with IPTG. The expression conditions were optimized by investigating different induction times, temperatures, and IPTG concentrations. The effect of these conditions on the solubility of the fusion protein was also investigated.



**Figure 4.1:** Schematic representation of the ligation of cDNA coding for PP14 into pUC18. *Sma I* indicates the cloning site into which the PP14 gene was inserted, *amp* indicates the gene coding for ampicillin resistance, *lacZ* indicates the  $\beta$ -galactosidase gene.



**Figure 4.2:** Schematic representation of the cloning of the gene for PP14 into the expression vector pGEX-KG. *EcoRI*, *HindIII* indicate the restriction sites into which the PP14 gene was cloned, *lacZ* indicates the  $\beta$ -galactosidase gene, *amp* indicates the ampicillin resistance gene, *ori* indicates the origin, *ptac* indicates the tac promoter gene, *lac* indicates the lac repressor, *gst* indicates the gene for glutathione-S-transferase

## 4.2 MATERIALS AND METHODS

The reagents, equipment and suppliers used in this study are listed in Appendix I.

### 4.2.1 Collection of tissue sample

An endometrium biopsy sample was taken from a healthy, fertile woman after informed consent had been obtained. The sample was taken during the luteal phase of the menstrual cycle timed from the date of the last period because this is when the endometrium is reported to be maximally synthesising and secreting PP14 (*Bell et al., 1987*). Thirty minutes before the sample was removed the patient was given a mild tranquilizer to act as a muscle relaxant. The sample was removed using a Sharman's curette from the fundus of the endometrium. The curette was inserted gently through the neck of the cervix and twisted round to remove the sample. The biopsy was placed into a small sterile container and snap frozen immediately in liquid nitrogen. It was then transferred to a -70°C freezer and stored until required.

### 4.2.2 Isolation of mRNA

All equipment and tubes in this part of the method were pre-treated with a 0.1% solution of diethyl pyrocarbonate (DEPC) for 2 hours at 37°C then rinsed thoroughly with RNase free water before use.

mRNA was extracted from the biopsy sample using the method of Chomczynski & Sacchi (*1987*). 100mg of biopsy sample was placed in a 1.5ml tube and kept on ice. 1ml of denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7, 0.5% sarkosyl, 100mM  $\beta$ -mercaptoethanol) was added and the tissue gently homogenised by drawing it up and down into a 1ml syringe through a 19 gauge needle until the tissue had broken down and a uniform suspension had been obtained. This tissue suspension was then split into 2 aliquots and each portion placed in a 1.5ml tube. 100 $\mu$ l of 2M sodium acetate buffer pH 4.0 was added to each tube. After mixing by inversion, 1ml of phenol saturated with water was added, the tubes were again mixed by inversion and 200 $\mu$ l of

chloroform:isoamyl alcohol mixture (49:1,v/v) was added. The tubes were shaken vigorously for 10 seconds, then chilled on ice for 20 minutes. After centrifuging at 10,000g for 20 minutes at 4°C the upper aqueous layers containing RNA were transferred to fresh tubes. An equal volume (650µl) of ice-cold isopropanol was added to each tube and the samples were cooled at -20°C for 1 hour to precipitate the RNA. The RNA was pelleted by centrifuging the tubes at 10,000g for 20 minutes at 4°C then the supernatant was removed and discarded. The pellets were each resuspended in 300µl of denaturing solution, then 300µl of isopropanol was added and after mixing the tubes were again cooled at 20°C for 1 hour. After centrifuging at 10,000g for 10 minutes at 4°C the supernatant was removed and discarded. 100µl of 75% ethanol was added without resuspending the pellet and the samples were frozen at -70°C until required for the reverse transcriptase reaction.

#### 4.2.3 Design of primers

Two synthetic oligonucleotides were designed from the published sequence of PP14 (*Julkunen et al., 1988*). *EcoRI* and *HindIII* sites were designed into the primers to enable the insertion of the PCR product into the multiple cloning site of the pGEX-KG expression vector

The primer sequences were:

5'-CGCGGAATTCGCATGCTGTGCCTCCTGCTC-3' specific for the 5' end incorporating an *EcoRI* site (underlined) and

5'-GCGCGAAGCTTCTAGAAACGGCACGGCTC-3' specific for the 3' end incorporating a *HindIII* site (underlined).

The absorbance of a diluted solution of each of the primers was measured at 260nm in a spectrophotometer. The extinction coefficient of the primers was calculated using the total molarity of the oligonucleotides and from these the molarities of the primer solutions were calculated.

#### 4.2.4 Reverse Transcription

The reverse transcriptase reaction was carried out using a First-strand cDNA synthesis kit (Pharmacia). One of the purified mRNA samples was thawed on ice, the 75% ethanol removed and the mRNA dried under vacuum in a rotary evaporator.

The pellet was resuspended in 8µl of DEPC treated water. The sample was estimated to contain 3mg total RNA. To this was added 5µl of bulk strand reaction mixture (containing reverse transcriptase, bovine serum albumin, dATP, dCTP, dGTP and dTTP in aqueous buffer), 1µl of 3' primer containing 20 pmol of DNA and 1µl of 200mM dithiothreitol (DTT). The reaction was incubated for 1 hour at 37°C.

#### 4.2.5 Polymerase chain reaction (PCR)

The cDNA obtained from the reverse transcriptase reaction was amplified using the polymerase chain reaction (PCR). The conditions were determined empirically and a variety of dilutions of cDNA were used as the template DNA.

PCR reactions were carried out in total volumes of 100µl. Each reaction consisted of 1µl of PP14 cDNA ( $1/1$ ,  $1/10$ ,  $1/100$ ,  $1/1000$ , dilutions), 2µl of 10mM dNTPs, 20 pmol of 3' primer, 20 pmol of 5' primer, 10µl 10x PCR buffer (100mM Tris-HCl, pH 9.0, 500mM KCl, 1% Triton X-100), 10µl of 25mM MgCl<sub>2</sub>, 3.75 units *Taq* Polymerase and Milli Q water to 100µl.

All the reagents except the *Taq* polymerase were assembled in a 500µl tube and heated to 100°C for 5-10 minutes. After cooling on ice for a few minutes the tubes were vortexed briefly and centrifuged at 10,000g, then the *Taq* polymerase was added and after gentle mixing the reaction mixture was overlayed carefully with 100µl of light mineral oil. The tubes were placed in the thermal cycler and the PCR reaction performed for 30 cycles. Each cycle consisted of a denaturing step at 94°C for 45 seconds, a primer annealing step at 50°C for 2 minutes and an extension step at 72°C for 90 seconds. The

tubes were then kept at 4°C. The mineral oil was removed by adding 100µl of ether to each tube and vortexing thoroughly. After centrifuging the tubes at 10,000g for 5 minutes the top layer was removed.

#### **4.2.6 Agarose gel electrophoresis**

The PCR reaction components were separated by electrophoresis on an agarose gel. A 1% gel was cast by melting 0.75g of agarose in 75ml of TAE buffer (40mM Tris-Acetate pH 8.0, 1mM EDTA) in a conical flask. The gel mixture was cooled to approximately 60°C by holding the flask under a running cold tap, then the gel was poured into a casting frame sealed at either end with masking tape. The samples were prepared by adding 1 volume of sample to 5 volumes of sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll, Type 400 in water). After the gel had set, 10µl portions of the samples were loaded. The size of the DNA fragments separated by electrophoresis was estimated by comparison with known molecular weight standards run on the same gel. 10µl of 225µg/ml bacteriophage λ DNA digested with *Hind*III or *Eco*RI and *Hind*III was run as molecular weight markers. The gel was run for 1 hour at 100V with TAE as running buffer. The gel was removed from the mould and placed in a container with 100ml of water containing 100µl of a 10mg/ml solution of ethidium bromide (EtBr) and gently agitated for 20 minutes. The EtBr solution was then washed off and the DNA visualised under UV light at 310 nm on a transilluminator and photographed.

#### **4.2.7 Purification of DNA**

The DNA was further purified using a 'GeneClean' kit. This method is based on the binding of DNA to glass beads.

The band containing the amplified DNA was carefully excised from the gel and placed in a pre-weighed 1.5 ml tube. The tube was reweighed and the weight of the gel calculated. 3mls of NaI solution (6M NaI, 160mM Na<sub>2</sub>SO<sub>3</sub>) was added for every gram of gel and the tubes were incubated at 50°C until the gel had dissolved (approximately 15



minutes). The tubes were then cooled on ice for 10 minutes. 5µl of 'glassmilk' was added and the tubes were left on ice for 15 minutes. During this time the tubes were vortexed every 5 minutes. The tubes were centrifuged for 5 seconds at 10,000g and the supernatants carefully removed. The pellet was resuspended in 500µl of 'newwash' (10mM Tris-HCl, pH 8.0, 10mM NaCl, 1mM EDTA in 75% Ethanol) and recentrifuged. The supernatant was again removed. This step was repeated a further 2 times. Finally the DNA was eluted from the glass beads by resuspending the pellet in 10µl of Milli Q water and incubating at 50°C for 5 minutes. After centrifuging for 10 seconds at 10,000g the supernatant was removed and retained and the resuspension, incubation and centrifugation was repeated. The second supernatant was pooled with the first.

#### **4.2.8 Formation of blunt ends and phosphorylation of PCR product**

The purified PCR product was ligated into pUC 18 using a Sureclone kit from Pharmacia.

The quantity of DNA obtained after purification using the 'GeneClean' kit was estimated by running an agarose gel of the sample with markers of known quantity of DNA and comparing visually the intensity of the bands obtained. The PCR product was prepared for ligation using a blunting/kinasing reaction. This reaction removes single-base 3' and 5' overhangs on the PCR product that may interfere with the subsequent ligation. At the same time the product is phosphorylated by T4 polynucleotide kinase. This reaction was carried out in a total volume of 20µl consisting of 16µl PCR product containing 100 ng of DNA, 1µl Klenow fragment, 2µl 10x buffer (660mM Tris-HCl, pH 7.6, 50mM MgCl<sub>2</sub>, 50mM dithiothreitol, 1mg/ml bovine serum albumin, 10mM hexaminecobalt chloride, 2mM ATP, 5mM spermidine HCl), and 1µl Polynucleotide Kinase.

The reaction mixture was incubated at 37°C for 30 minutes then 20µl of phenol/chloroform was added and the mixture was vortexed then centrifuged at 10,000g for 1 minute. The upper layer was removed and purified by centrifugation at 10,000g for 30 seconds through an Sephacryl S-200 Microspin column.

#### 4.2.9 Ligation into pUC 18

The purified blunt-ended DNA was ligated into pUC 18. This vector was provided in the SureClone kit pre-digested at the *Sma* I site and dephosphorylated. The ligation reaction was carried out in a total volume of 20µl and consisted of 10ng DNA, 10µl 10x ligation buffer (300mM Tris-HCl, pH 7.8, 100mM MgCl<sub>2</sub>, 100mM dithiothreitol, 10mM ATP), 50 ng of pUC 18 vector DNA, 1µl DTT (200mM solution), 1 unit of T4 Ligase, Milli Q water up to 20µl. The reaction was carried out for 2 hours at 16°C.

A control ligation reaction treated in an identical manner contained all the reagents except the DNA which was replaced by water. After the ligation the reactions were placed on ice until required for the transformation of *E. coli*.

#### 4.2.10 Preparation of competent *E. coli* JM109 cells

Competent cells were prepared by the method of Nishimura et al. (1990). 5ml of Luria Broth (LB) medium (10g tryptone, 5g yeast extract and 10g NaCl in 1 litre H<sub>2</sub>O) was inoculated from an original stock of *E. coli* JM109 cells. The cells were grown overnight at 37°C with vigorous shaking in a loosely capped culture tube. A 100ml flask containing 50ml of LB containing 10mM MgSO<sub>4</sub> and 0.2% glucose (medium A) was inoculated with 500µl of the overnight culture. The flask was shaken vigorously at 37°C with good aeration for 2½ hours. The cells were placed on ice for 10 minutes then pelleted by centrifuging at 1500g for 10 minutes at 4°C. The cells were resuspended gently in 500µml of medium A and 2.5ml of a storage medium of LB containing 36% glycerol, 12% PEG 8000 and 12mM MgSO<sub>4</sub> and 100µl aliquots were placed in 1.5ml eppendorf tubes. The tubes were stored at -70°C until used. The viability of the cells was tested before using them for transformations by streaking a plate of LA (LB with 15g of agar added per litre) with the cells and observing the growth at 37°C overnight.

#### **4.2.11 Transformation of *E.coli***

Competent JM 109 cells were transformed by removing them from the -70°C freezer and placing the tubes immediately on ice. The cells were allowed to thaw on ice for a few minutes, and 5µl of the ligation mixture added. Aliquots of cells containing the control ligation mixture without the insert, 1µl of the vector only and no DNA at all were also prepared as controls. The tubes were gently mixed and left on ice for 15-30 minutes. The cells were then heat-shocked by placing them in a 42°C water bath for exactly 2 minutes. After replacing the tubes on ice for 2 minutes 1ml of pre-warmed LB was added to each tube, the tubes were gently mixed by inversion and the cells were incubated at 37°C for 45-60 minutes. The tubes were centrifuged at room temperature for 15-40 seconds at 10,000g and most of the supernatant was removed to leave 200µl of medium in the tube. At this point for transformations of pUC 18 40µl of 200mM isopropyl-thio-β-D-galactoside (IPTG) and 40µl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) dissolved in dimethylformamide was added to each tube of cells. Transformations of pGEX-KG as described later did not have any further reagents added to the cells, since this plasmid does not have blue/white selection. Both pUC 18 and pGEX-KG select for ampicillin resistance so the cells were gently resuspended in the remaining medium and plated onto pre-warmed L<sub>amp</sub> plates. 1g of agar was dissolved in 100mls of LB, the mixture was autoclaved and after cooling to 60°C, ampicillin was added to make a final concentration of 50µg/ml. 25ml aliquots were dispensed into petri dishes and left to set. After plating out the transformed cells the plates were incubated overnight at 37°C.

#### **4.2.12 Identification of transformants**

The positive colonies could be identified from those containing only religated vector due to their inability to make β-galactosidase and convert X-Gal to a blue product. The production of β-galactosidase may cause the colonies containing this activity to grow slower than the white colonies positive for the insert, and appear as pinpoints rather than fully developed colonies, therefore some colonies that are in fact negative for the insert

can initially appear as white positive colonies. The blue colour can be enhanced by placing the plates at 4°C for a few hours. After this incubation 36 white colonies from the plate with the ligation mixture containing the insert were picked off the plate using an inoculation loop and grown up overnight in 5ml of L<sub>amp</sub> broth (LB containing 50µg/ml ampicillin) at 37°C with in loosely capped culture tubes with vigorous shaking.

#### **4.2.13 Minipreparation of plasmid DNA**

Minipreparations of DNA were prepared as described in Maniatis (*Maniatis et al., 1980*). 1.5ml of each overnight culture was placed in a 1.5ml tube and the cells pelleted by centrifuging for 1 minute at 10,000g. The cells were resuspended by vigorous vortexing in 100µl of ice-cold miniprep I solution (25mM Tris-HCl pH 8.0, 50mM glucose, 10mM EDTA). After leaving the tubes at room temperature for 5 minutes with the lids open 200µl of freshly made miniprep II solution (0.2M NaOH, 1% SDS in H<sub>2</sub>O) was added, the tubes mixed by inversion and placed on ice for 5 minutes. Following this, 150µl of ice-cold miniprep III solution (60ml 5M potassium acetate, 11.5ml acetic acid, 28.5ml water) was added and the tubes again left on ice for 5 minutes. After briefly vortexing the tubes were centrifuged for 5 minutes at 10,000g and the supernatants transferred to fresh tubes. An equal volume (450µl) of phenol was added, the tubes were vortexed thoroughly and centrifuged for 5 minutes at 10,000g. The supernatants were transferred to fresh tubes and 500µl of ether was added. The tubes were again vortexed and centrifuged at 10,000g for 2 minutes. The supernatants were removed and discarded and 1ml of ethanol at -20°C was added. The tubes were left on ice for 10 minutes, then centrifuged for 7 minutes at 10,000g. The supernatants were removed and discarded and the pellet was washed with 100µl of 70% ethanol. The pellet was dried using a rotary evaporator centrifuge and then resuspended in 20µl of Milli Q water.

#### **4.2.14 Restriction digests**

Plasmid DNA from the minipreps was digested with restriction enzymes to cut out the PCR product. The reaction mixture was carried out in a total volume of 20µl containing 12 units of *EcoRI*, 12 units of *HindIII*, 2µl of 10x incubation buffer (100mM Tris-HCl

pH 7.5, 50mM NaCl, 6mM MgCl<sub>2</sub>, 6mM β-mercaptoethanol), 1μl of 1mg/ml RNase A (previously boiled for 15 minutes to remove DNase activity), 10μl of miniprep DNA and Milli Q water to make the volume up to 20μl. The reaction was carried out at 37°C for 1 hour and then 10μl of the reaction mixture was electrophoresed on an agarose gel as before.

Clones that potentially contained the insert for PP14 were identified from the gel by the presence of two bands, one with approximate size of the insert (543 base pairs) in addition to the plasmid band (5000 base pairs). Clones that did not contain an insert were identified by the presence of only one band.

#### **4.2.15 Sequencing of DNA**

The sequence of the insert now in the plasmid pUC 18 in the positive clones was checked using a USB sequencing kit. This was to confirm that the correct piece of DNA had been ligated and that the sequence agreed with the published sequence.

Three additional primers were used for sequencing, 2 were from the kit and were designed to be used to sequence DNA fragments in the M19 range of vectors. The third primer was designed from the centre of the PP14 sequence.

For each of the primers (5 in total) 7.5 μl of a miniprep containing 4μg of DNA was denatured by adding 2μl of 2M NaOH, vortexing and centrifuging at 10,000g briefly then incubating at room temperature for 10 minutes. 3μl of 3M sodium acetate and 7μl of Milli Q water were then added and the DNA was precipitated by the addition of 60μl of 100% ethanol. After a 30 minute incubation at 70°C the DNA was centrifuged at 10,000g for 10 minutes, the pellet was washed with 70% ethanol and then resuspended in 10μl of Milli Q water.

Sequencing reactions: 10μl of template DNA was annealed to 1pmol of each primer in 2 μl with 2μl of annealing buffer. After vortexing and centrifuging at 10,000g briefly the

reaction tube was heated at 65°C for 5 minutes, then 37°C for 10 minutes. The tubes were then cooled to room temperature for at least 10 minutes, then centrifuged at 10,000g briefly. For each primer 4 tubes were prepared labelled 'A', 'C', 'G' and 'T'. 2.5µl of the A,C,G and T mixture were dispensed into the appropriate tubes. These tubes were pre-warmed at 37°C for at least 1 minute. An 'enzyme pre-mix' was prepared by mixing 2µl of T7 DNA Polymerase, 14µl of enzyme dilution buffer, 3µl of labelling mix, 13µl of water, 8µl of DTT and 8µl of <sup>32</sup>P-dATP and kept on ice. 6µl of this mix was added to each annealed template and primer to carry out the labelling reaction. After mixing gently the tubes were incubated at room temperature for 10 minutes. 4.5µl of each labelling reaction was added to each of the tubes labelled A,C,G and T. After 5 minutes incubation at 37°C the reactions were stopped by the addition of 5µl of stop solution. The tubes were then stored on ice until they were loaded onto the gel. Just prior to loading the gel the reactions were heated to 75-80°C for 2 minutes.

The sequencing gel was prepared by washing the plates with water, then ethanol, then coating them with silicone. Spacers were placed across the bottom and down either side and then plates were held together with clamps. The gel mixture was prepared by gently heating 5.7g acrylamide, 0.3g bisacrylamide and 42g of urea in TBE (100mM Tris-Borate, 2mM EDTA) until the mixture was dissolved. After cooling to room temperature the mixture was filtered through a 0.2µm filter, topped up to 100 ml with TBE. Immediately before use 1 ml of fresh 10% ammonium persulphate was added and then swirled to mix. 40µl of TEMED was then added and the gel mixture poured carefully into the prepared plate sandwich. The comb was inserted at the top and the gel left to set for at least 1 hour.

The gel was placed in the apparatus and the comb removed. TBE was washed along the top of the gel to remove the urea and the comb reinserted to form 40 wells across the gel. The gel was pre-run at 1800V for 1 hour prior to loading the samples. The gel was run in TBE buffer. 2.5µl of each of the 20 samples was loaded in the order A, C, G, T

and the gel run for 2 hours. The samples were then reapplied to the remaining 20 wells and the running of the gel continued for a further 2 hours.

After running the gel was transferred onto filter paper and exposed to autoradiography film for 2 hours at -70°C in the dark.

#### **4.2.16 Preparation of insert from positive colonies**

5ml cultures of the positive clones were grown up in  $L_{amp}$  broth and the DNA prepared as before. The DNA was digested with restriction enzymes in a total volume of 80µl consisting of 40µl miniprep DNA, 36 units of *Eco*RI, 36 units of *Hind*III, 8µl 10x buffer (100mM Tris-HCl pH 7.5, 50mM NaCl, 6mM  $MgCl_2$ , 6mM β-mercaptoethanol), made up to 80µl with Milli Q. The reaction was carried out at 37°C for 1½ hours.

The DNA was purified by running an agarose gel and the band containing the insert was excised and treatment with the 'GeneClean' kit as described before.

#### **4.2.17 Preparation of pGEX-KG vector**

The original pGEX-KG vector was a kind gift of Dr. A Bannister. The vector used to ligate the PP14 insert into was a modified version (pHZP3GEX1.1). This contains the insert hZP3 which codes for the human zona pellucida protein ZP3 ligated into the *Eco*RI and *Hind*III sites.

An overnight culture of JM 109 containing the pHZP3GEX1.1 vector was grown up in 2 x 5ml loosely capped culture tubes overnight at 37°C with vigorous shaking. Miniprepations of plasmid DNA were made as described before.

#### **4.2.18 Restriction digestion of pHZP3GEX1.1**

The DNA was digested with restriction enzymes in a total volume of 80µl consisting of 40µl DNA, 36 units of *Eco*RI, 36 units of *Hind* III, 8µl 10x buffer (100mM Tris-HCl pH 7.5, 50mM NaCl, 6mM  $MgCl_2$ , 6mM β-mercaptoethanol), Milli Q water up to 80µl.

The reaction was carried out for 1½ hours at 37°C and then separated on an agarose gel and the band containing the plasmid carefully excised. The DNA was purified using the 'GeneClean' kit as before.

#### **4.2.19 Ligation of the insert into pGEX-KG**

The PP14 insert was ligated into the pGEX-KG vector in a total volume of 20µl consisting of 1µl pGEX containing 12ng of DNA, 1µl insert containing 16ng of DNA, 1 µl 10x ligation buffer (300mM Tris-HCl, pH 7.8, 100mM MgCl<sub>2</sub>, 100mM DTT, 10mM ATP), 2.5 units of T4 DNA ligase, Milli Q water to 20µl.

The reaction was carried out at 4°C overnight. Control reactions were also carried out as described before. Competent cells were transformed with 5µl of each ligation mixture and L<sub>amp</sub> plates inoculated as described before.

#### **4.2.20 Identification of transformants**

After overnight incubation at 37°C the plates were inspected for colonies. When cloning into pGEX-KG the incidence of false positives is much higher than in pUC 18 since there is no way of visually screening between positive and negative religated vector. 36 colonies were picked off the plate inoculated with the cells containing the ligation mixture with the insert. These colonies were expanded in 5ml overnight cultures and minprepped and digested as described before. The potentially positive clones were identified by running a gel and looking for a band corresponding to the size of the insert.

#### **4.2.21 Transformation of positive recombinant clones with plasmid pUBS250**

Positive clones containing the PP14 insert in pGEX-KG were used to prepare competent cells as previously described. The cells were transformed as described by the addition of 1µl of the plasmid pUBS250 which codes for the *argU* gene. This time the cells were plated onto twin selection plates containing 25µg/ml ampicillin and 25µg/ml kanamycin,



since the pUBS250 plasmid selects for kanamycin resistance. Colonies exhibiting kanamycin resistance were assumed to contain the pUBS250 plasmid.

#### **4.2.22 Expression of recombinant protein**

5ml cultures of positive clones were grown overnight at 37°C with shaking and good aeration in  $L_{amp/kana}$  broth (LB containing 25µg/ml ampicillin and 25µg/ml kanamycin). 50µl of this overnight culture was inoculated into 5ml of fresh  $L_{amp/kana}$  broth. The cultures were grown up under the same conditions for 2 hours and 1ml of uninduced culture removed. Protein expression was induced by the addition of IPTG to a final concentration of 1mM. 1ml of culture medium was removed every hour for 3 hours after induction. Control cultures with the plasmids pGEX-KG and pUBS250 without the insert were also induced. Control cultures of *E. coli* JM109 cells were also grown up in LB without antibiotics and induced. The samples were spun for 1 minute to pellet the cells and total cell extracts were prepared by resuspending the cells in 100µl SDS-PAGE running buffer (50mM Tris-HCl, pH 6.8, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). SDS-PAGE was carried out as described in appendix IV.

#### **4.2.23 Protein solubility**

The solubility of the expressed protein was investigated by growing up further 5ml cultures of the positive clones and inducing them for 3 hours as before. 1ml of cells were pelleted and resuspended in 100µl of PBS and sonicated on ice for 2 bursts of 10 seconds each at an amplitude of 10µ. The suspension was then centrifuged at 10,000g for 30 minutes and the pellet resuspended in SDS-PAGE running buffer as before. SDS-PAGE was run on the resuspended pellets and the supernatants to establish the proportion of expressed protein in the soluble fraction.

#### **4.2.24 Optimisation of expression conditions**

From the results of the previous experiment it was apparent that a substantial proportion of the expressed protein was in an insoluble form when expressed under the initial

growth conditions described. A number of changes were made to increase the proportion of expressed protein in the soluble fraction. The growth temperature was investigated since it has been reported that lower expression temperatures increase the solubility of some pGEX-KG fusion proteins (*Taylor et al., 1993*). Cultures were grown as before for 3 hours at 37°C, 30°C and 25°C. Cells were sonicated as before and the supernatants and resuspended pellets run on SDS-PAGE.

The effect of the sarkosyl concentration on solubility was also investigated. Cell pellets were sonicated in 0.05%, 0.1% and 0.5% solutions of sarkosyl. The soluble and insoluble fractions obtained were electrophoresed and the amount of expressed protein in the soluble fraction assessed.

#### **4.2.25 Scale-up of protein expression**

The expression of recombinant PP14 was scaled up into 100ml flasks. Overnight cultures of the positive clones were grown in 10 ml aliquots of L<sub>amp/kana</sub> at 37°C. Flasks containing 100ml L<sub>amp/kana</sub> (up to 10 flasks) were each inoculated with 1ml of overnight culture. The flasks were placed on a shaker at 37°C for 2 hours. The flasks were then cooled on ice for 10 minutes before IPTG at a final concentration of 0.05mM was added. The cultures were induced at 25°C with shaking. The induction time was increased to 4 hours to take account of the slower growth rate of the cells at 25°C. The cells were harvested at 4°C by centrifuging at 3000g for 10 minutes, and each pellet was resuspended in 1ml sonication buffer (PBS, 0.5% sarkosyl, 0.1mM PMSF). The cell pellets were each sonicated on ice for a total of 1 minute in 10 second bursts with 2 minutes rest on ice between each sonication. The suspensions were then centrifuged at 10,000g for 30 minutes at 4°C and the supernatants pooled.

#### **4.2.26 Affinity purification of fusion protein**

The fusion protein was purified by a modification of the method of Guan & Dixon (*1991*). The supernatant was applied to a 2ml glutathione-agarose column previously equilibrated with 40mls of ice-cold PBS. The column flow was stopped and the resin

resuspended, and left on ice to resettle for 20 minutes. The flow was restarted and the eluent collected and then reappplied to the column. The column was then washed with 40mls of ice-cold PBS, followed by 20mls of thrombin cleavage buffer (Tris-HCl, pH 8.0, 150mM NaCl, 2.5mM CaCl<sub>2</sub>).

#### **4.2.27 Western blotting**

The identity of the band at 44kD as the fusion protein was confirmed by western blotting as described in appendix III. The nitrocellulose membrane was probed with polyclonal rabbit antibodies against glutathione *S*-transferase at a dilution of 1/30,000 and against PP14 at a dilution of 1/1000.

## 4.3 RESULTS

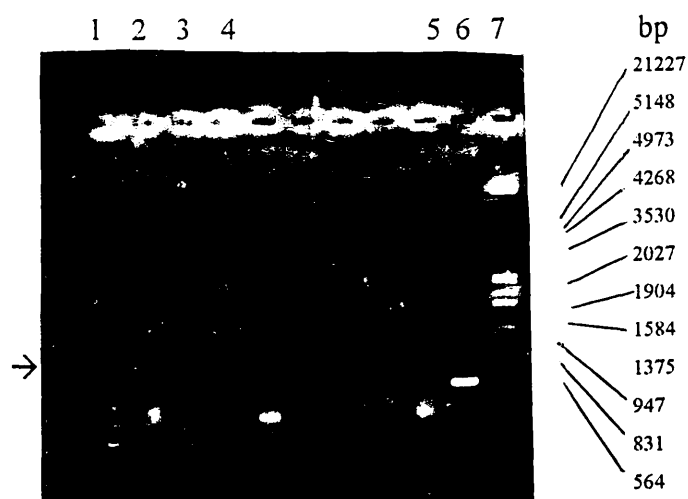
### 4.3.1 Cloning of cDNA for PP14

mRNA was extracted from endometrial tissue and used in a reverse transcriptase reaction and then PCR. Figure 4.3 shows the results of the polymerase chain reaction. A band was obtained at approximately 546 bps, assumed at this point to be DNA coding for PP14, this reaction was carried out a number of dilutions of cDNA, as indicated on the figure. Positive results were obtained at dilutions of  $1/1$ ,  $1/100$ , and  $1/1000$ . The reaction at a dilution of  $1/10$  did not give a positive result, the reason for this is not known.

The band observed at 546 bps was cut out of the gel and used to prepare DNA to ligate into the cloning vector pUC18. The results of the ligation are shown in Figure 4.4. Two bands were obtained, one at 546 bps, presumed to be PP14 and the plasmid band at 2635bps. Again the insert was cut out of the gel and used to prepare DNA to ligate into the expression vector pGEX-KG.

DNA was prepared from the PP14 clone in pUC18 and sequenced to confirm that the insert definitely coded for PP14. Part of the sequencing gel obtained is shown in figure 4.5. The results from this experiment demonstrated that cDNA coding for PP14 had been successfully cloned and that the sequence obtained agreed with the published sequence.

The method used to ligate the PP14 cDNA into pGEX-KG was slightly unusual. Several attempts to ligate the PP14 insert into pGEX-KG digested with *EcoRI* and *HindIII* were unsuccessful with no apparent reason for this failure discovered. A clone containing the gene coding for the zona pellucida binding protein ZP3 ligated into these sites in pGEX-KG was available in the laboratory. This clone was digested, run on a gel and the



**Figure 4.3:** Agarose gel of PCR reactions. cDNA coding for PP14 was amplified at different dilutions. The bands presumed to code for PP14 are indicated.

Lane 1: PP14 cDNA  $1/1$

Lane 2: PP14 cDNA  $1/10$

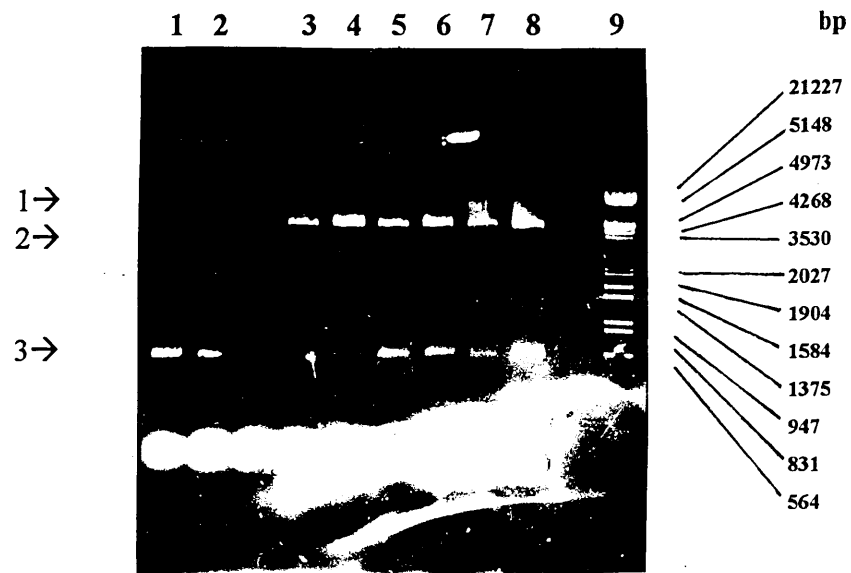
Lane 3: PP14 cDNA  $1/100$

Lane 4: PP14 cDNA  $1/1000$

Lane 5: PP14 cDNA  $1/1$

Lane 6: Control PCR reaction

Lane 7: Molecular weight markers,  $\lambda$ DNA cut with *EcoRI* and *HindIII*

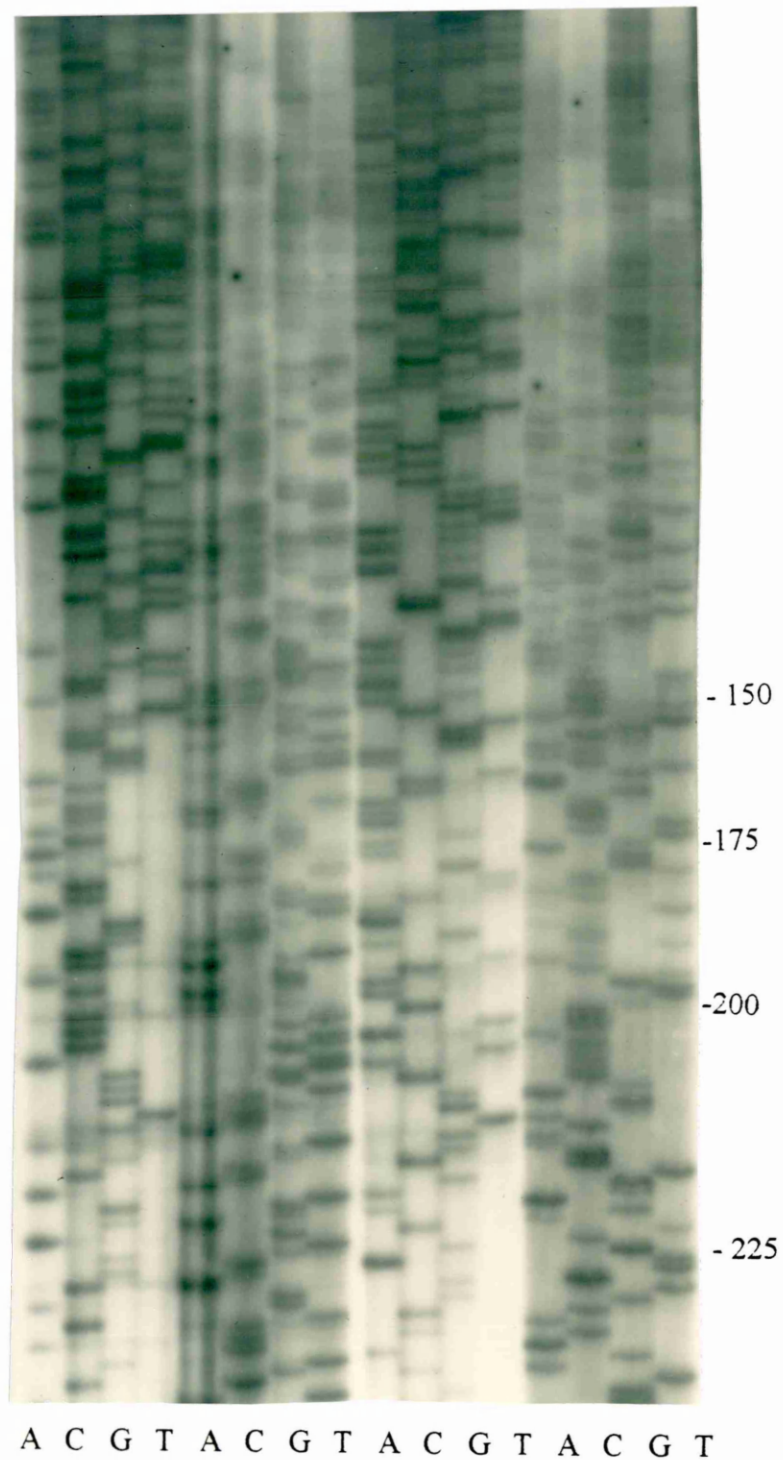


**Figure 4.4:** Agarose gel of digested plasmids pPP14UC 18 and pPP14GEX-KG containing the PP14 insert. The plasmids and PP14 insert are indicated by arrows (1: pGEX-KG, 2: pUC 18, 3: PP14). The plasmids were digested with *EcoRI* and *HindIII*.

Lanes 1 and 2: pPP14UC 18

Lanes 3,4,5,6,7,and 8: pPP14GEX.KG

Lane 9: Molecular weight markers,  $\lambda$ DNA digested with *EcoRI* and *HindIII*.



**Figure 4.5:** Part of the sequence of PP14 as analysed by sequencing gel. The region sequenced in the four lanes to the extreme right corresponds to base pairs 150-225 as indicated.

plasmid band cut out and used to prepare plasmid DNA to ligate with the PP14 cDNA. The ligation reaction worked at the first attempt. The results of this ligation are shown in figure 4.4 alongside the results from the ligation into pUC18.

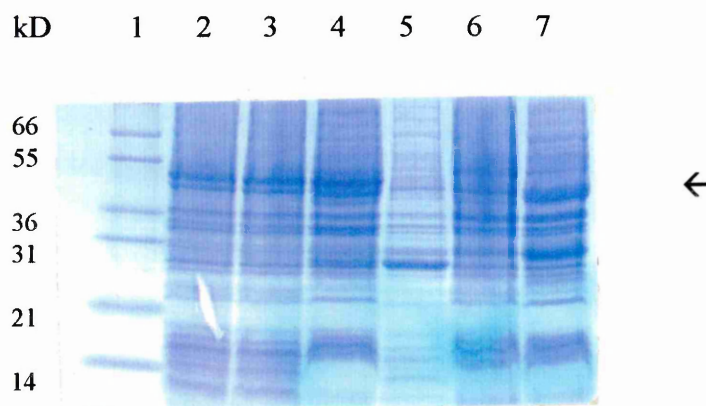
#### **4.3.2 Expression of recombinant PP14**

Having obtained a recombinant clone for PP14 the expression of the protein was investigated. The fusion protein was induced by the addition to small-scale cultures of IPTG. Control cultures containing JM109 cells alone and pGEX-KG only without the PP14 gene were grown up alongside the PP14 clone. All cultures were controlled with non-induced cultures. The cells from these cultures were lysed, and samples run on SDS-PAGE. Figure 4.6 show the results obtained from the induction of GST-PP14. An additional band can be seen in the lane containing the induced PP14 clone at 44kD. The molecular weight of GST is 26kD and that of the PP14 monomer is predicted at 18,787 (*Julkunen et al., 1988*), giving a predicted fusion protein weight of 45 kD, therefore the observed molecular weight of the induced band agrees well with the expected result. There is a clearly induced band at 26kD in the lane containing pGEX-KG only, corresponding to GST.

#### **4.3.3 Optimisation of expression**

The solubility of expressed recombinant proteins is often a problem therefore the effect of various conditions on the solubility of recombinant PP14 was investigated. Three different expression temperatures were compared and the concentration of the solubilisation reagent sarkosyl was also studied. Several small-scale cultures were grown at each temperature and the cells sonicated and centrifuged in different concentrations of sarkosyl. The results of these experiments are shown in figure 4.7. Dropping the temperature from 37°C improved the solubility, in the top gel lanes 1-6 are of cells grown at 37°C, only lane 2 at the top concentration of sarkosyl shows significant soluble protein, and this is less than half the expressed protein. In comparison the lanes containing cell supernatants from the cultures grown at 30°C (top gel lanes 8-10 and





**Figure 4.6:** SDS-PAGE gel of induced GST-PP14 fusion protein

Lane 1: Molecular weight markers

Lane 2: JM109 cells uninduced

Lane 3: JM109 cells induced with IPTG

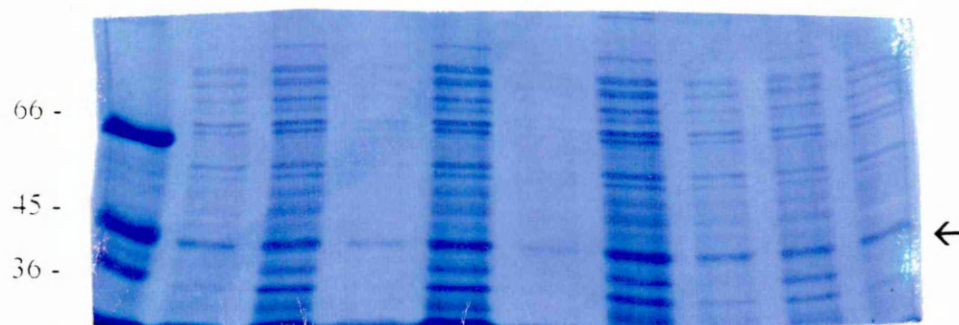
Lane 4: pGEX.KG uninduced

Lane 5: pGEX.KG induced with IPTG

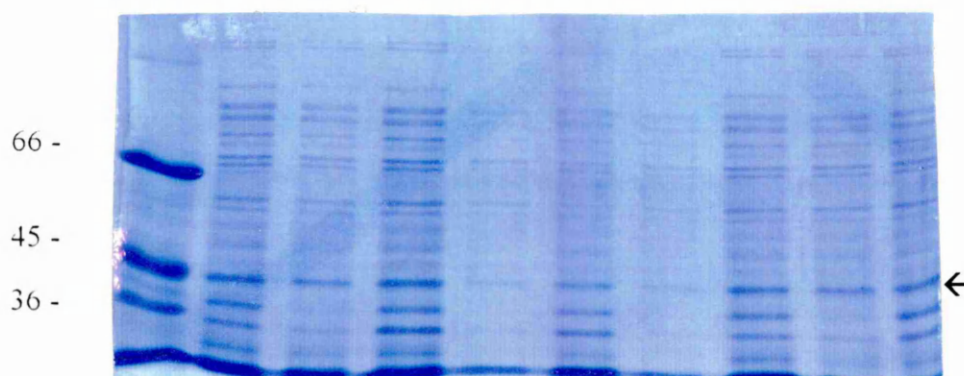
Lane 6: pPP14GEX.KG uninduced

Lane 7: pPP14GEX.KG induced with IPTG

PP14-GST is indicated by the arrow at 44kD



1 2 3 4 5 6 7 8 9 10



**Figure 4.7:** SDS-PAGE gels of optimisation of temperature of expression and sarkosyl concentration in the expression of the fusion protein GST-PP14.

Top gel:

Lane 1: Molecular weight markers  
 Lane 2: 37°C, 0.5% sarkosyl, supernatant  
 Lane 3: 37°C, 0.5% sarkosyl, pellet  
 Lane 4: 37°C, 0.1% sarkosyl, supernatant  
 Lane 5: 37°C, 0.1% sarkosyl, pellet  
 Lane 6: 37°C, 0.05% sarkosyl, supernatant  
 Lane 7: 37°C, 0.05% sarkosyl, pellet  
 Lane 8: 30°C, 0.5% sarkosyl, supernatant  
 Lane 9: 30°C, 0.5% sarkosyl, pellet  
 Lane 10: 30°C, 0.1% sarkosyl, supernatant

Bottom gel:

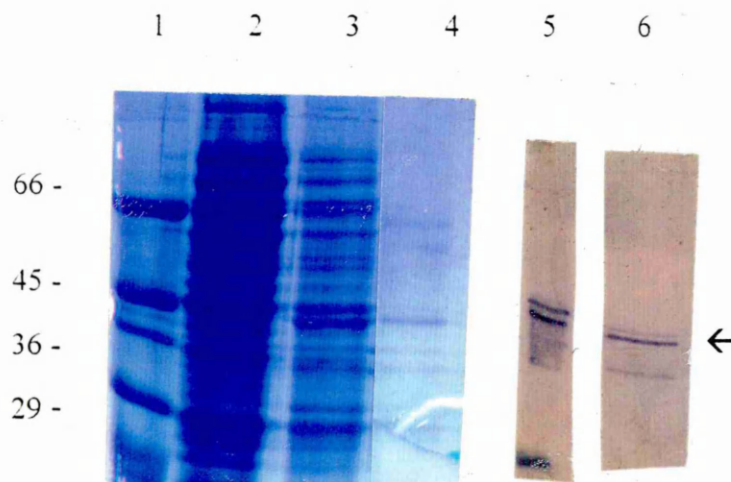
Lane 1: Molecular weight markers  
 Lane 2: 30°C, 0.1% sarkosyl, pellet  
 Lane 3: 30°C, 0.05% sarkosyl, supernatant  
 Lane 4: 30°C, 0.05% sarkosyl, pellet  
 Lane 5: 25°C, 0.5% sarkosyl, supernatant  
 Lane 6: 25°C, 0.5% sarkosyl, pellet  
 Lane 7: 25°C, 0.1% sarkosyl, supernatant  
 Lane 8: 25°C, 0.1% sarkosyl, pellet  
 Lane 9: 25°C, 0.05% sarkosyl, supernatant  
 Lane 10: 25°C, 0.05% sarkosyl, pellet

bottom gel lanes 2-4) and 25°C (bottom gel lanes 5-10) both show higher proportions total expressed protein in the soluble fraction. At 30°C the greatest solubility was obtained at sarkosyl concentrations of 0.5% and 0.1%, but at 25°C the expressed protein could be solubilised at a sarkosyl concentration of 0.05%. At 25°C the higher two sarkosyl concentrations did not appear to solubilise the protein effectively, the reason for this is not known, however higher concentrations of sarkosyl can lead to micel formation and so choosing a lower concentration may be desirable. 25°C was therefore chosen as a optimum expression temperature, and 0.05% sarkosyl as the detergent concentration.

#### **4.3.4 Purification of fusion protein**

100ml volumes of cells were grown up at 25°C then centrifuged, resuspended in 0.05% sarkosyl, sonicated and recentrifuged. The supernatants obtained were then applied to a glutathione-agarose column. Figure 4.8 shows the results from an SDS-PAGE gel of this purification and a Western blot of some of the lanes of the gel probed with polyclonal antibody against GST. It can be seen from this gel that this purification method does not succeed in obtaining a pure preparation of fusion protein. There appear to be several other bands that co-purify. This may be the results of aggregates in the case of the higher molecular weight species and truncated protein expression in the case of the lower molecular weight species. The Western blot clearly shows the fusion protein band, and also picks up a few of the other bands confirming that they contain GST and therefore may be truncated fusion protein.

The GST expression system is designed to allow the straightforward cleavage of the expressed protein from its fusion partner GST with thrombin. In practice this reaction appears to be inefficient, and despite several attempts the cleavage reaction of GST from PP14 was unsuccessful. This problem has been encountered by other users of the GST expression system (*Taylor et al., 1993*).



**Figure 4.8:** SDS-PAGE gel of purification of fusion protein GST-PP14 using glutathione-agarose. Western blot of GST-PP14 fusion protein.

Lane 1: Molecular weight markers

Lane 2: Pellet after sonication

Lane 3: Supernatant after sonication

Lane 4: Supernatant after elution from glutathione-agarose column

Lane 5: Western blot of Lane 3

Lane 6: Western blot of Lane 4

#### 4.4 DISCUSSION

cDNA coding for PP14 has been cloned and over-expressed in *E. coli* as a fusion product with GST. cDNA was prepared from mRNA from endometrial tissue and amplified using the PCR reaction. The gene obtained was then ligated into pUC18 followed by a cross-over ligation reaction into the expression vector pGEX-KG. This vector expressed PP14 as a fusion protein with GST in response to induction with IPTG. This fusion protein could be solubilised when grown at 25°C and resuspended in 0.05% sarkosyl. Although the fusion protein was partially purified using glutathione agarose, the cleavage reaction to separate the two proteins proved unsuccessful. This may have been due to protein aggregation, masking the cleavage site. The activity of this recombinant protein has not yet been studied due to the failure to obtain a pure preparation isolated from GST. However modifications to the cleavage procedure may yield isolated protein and then activity studies can be carried out. An additional factor to take into account when investigating recombinant PP14 is the possible requirement for a cofactor for activity. The next chapter considers this problem.

The availability of recombinant PP14 facilitates the possible future therapeutic use of this protein. It will also provide a source of protein for further investigations into the activity of this molecule.

## **4.5 CONCLUSION**

These results describe the successful cloning and expression of PP14 as a recombinant protein. This provides a basis for further investigations into the clinical application of PP14, and the activity of this molecule.

# CHAPTER FIVE: MOLECULAR MODELLING OF PP14 STRUCTURE

## 5.1 INTRODUCTION

Placental protein 14 (PP14) is a member of the lipocalin family of proteins. At present over 20 members of this family have been characterised, and with the help of computerised sequence analysis programs that identify sequence homology between molecules, the number of members assigned to the family is increasing steadily.

The first member was retinol-binding protein (RBP). The three-dimensional structure of RBP was resolved by X-ray crystallography revealing a novel folding pattern and it was suggested that it might be a member of a new family of proteins (*Newcomer et al., 1984*). When the structure of  $\beta$ -lactoglobulin ( $\beta$ LG) was determined a similar folding pattern was found (*Godovac-Zimmermann et al., 1985; Sawyer et al., 1985*), in fact the two structures can be super-imposed on each other, and this observation, together with some regions of sequence homology identified  $\beta$ LG as a second member of this new group of proteins (*Papiz et al., 1986*).

It was already known that  $\beta$ LG binds retinol (*Fugate & Song, 1980*) so this implied that the family was a group of retinol-binding molecules. However the next proteins identified as members were  $\alpha$ -1-microglobulin (*Pervaiz & Brew, 1985*),  $\alpha$ -2-microglobulin and bilin-binding protein (*Huber et al., 1987; Holden et al., 1987*), which shared structural features with RBP and  $\beta$ LG but have very different ligand specificity. This widened the description of the proposed role of the lipocalins to that of a family of adaptable carrier molecules consisting of a group of proteins of similar size (150-200 amino acid residues), (*North, 1989*), which share a common three-dimensional structure despite low overall sequence homology.

When the sequence of PP14 was published (*Julkunen et al., 1988*) the presence of regions of sequence homology identified it as a lipocalin.

### 5.1.1 Structural features

There are three features that identify a protein as a lipocalin: the characteristic folding pattern of the three-dimensional structure; the presence of a binding pocket or calyx lined with predominately hydrophobic residues; and the presence of specific, characteristic regions of sequence conservation. Analysis of the three-dimensional structure of RBP,  $\beta$  LG and major urinary protein (MUP) shows a conserved  $\beta$ -barrel structural motif of eight anti-parallel  $\beta$ -strands in a criss-cross pattern arranged as two  $\beta$ -sheets stacked on top of one another. This forms a compact, protease-resistant framework, surrounding a hydrophobic core which provides a protected binding site for labile and water insoluble hydrophobic molecules.

In addition there is a conserved  $\alpha$ -helix motif at the carboxy end of the proteins, and a disulphide bridge linking this helix to the centre of the  $\beta$ -strands. The amino acid sequences of the lipocalins vary widely with no more than 30% sequence homology between any two members of the family (*Åkerström & Lögdberg, 1990*), but there are certain regions of the sequences that are highly conserved (*Godovac-Zimmerman, 1988; Perviaz & Brew, 1985*). The sequence Gly-X-Trp (where X is any amino acid) is conserved near residue 25 in all lipocalins identified so far (*Nagata et al., 1991*) with regions on either side sharing some homology. In addition the sequence Thr-Asp-Tyr is conserved near residue 100 in the majority of lipocalins, though it is not universal, again with some further homology in the flanking regions. The Gly-X-Trp set of residues form part of the first  $\beta$  strand and the Thr-Asp-Tyr set form part of a loop between the sixth and seventh  $\beta$  strands (*Godovac-Zimmermann, 1988*), this loop crosses the same region in the three-dimensional structure as the first strand, forming a specific area in the molecule which is highly conserved. The Trp residue at 28 in  $\beta$ LG, 23 in MUP and 24 in RBP, is at the back of the proposed binding pocket, in the centre of this highly conserved region and has been suggested as the crucial residue in the binding site (*Papiz et al., 1986*). An arginine residue around residue 125 positioned behind the tryptophan at the base of the binding pocket is another highly conserved feature.



### 5.1.2 Receptors and specificity

The mammalian lipocalins have associated receptors (*Sawyer, 1987*), and the conserved area of the molecular structure has been proposed as the receptor binding site. However the RBP receptor has been described as interacting with the opening on the binding pocket which is on the opposite side of the molecule from the conserved area (*Sivaprasadarao et al., 1993*). There is wide sequence variation between lipocalins in this area of the molecule which would ensure specificity for the receptor. Therefore there appears to be a common protein structural framework with specific modifications to give the selectivity for different ligands (*Godovac-Zimmerman, 1988*). The variations to give the specificity may be at the opening to the binding pocket, which may also serve as a receptor binding site, or within the binding pocket, to select only the desired ligand. The area of sequence conservation may be a binding site common to all lipocalins, with variations to the surrounding sequences rendering the site specific for a particular receptor. The mode of action may be that a conformational change occurs upon ligand binding, enabling the lipocalin to bind to a receptor that it cannot bind without the ligand, and passing on a signal to the target cell, or the mechanism may be a ligand delivery system, and once the lipocalin is bound to the receptor the ligand is passed to the receptor, probably via the tryptophan and arginine residues at the back of the binding pocket.

### 5.1.3 Ligand binding

Individual members of the family bind a range of different molecules in the core region of their structure, these molecules include Vitamin A (retinol-binding protein), biliverdin (insectocyanin/bilin-binding protein) and pheromones (major urinary protein). The common pattern is the binding of hydrophobic molecules in an aqueous environment. There are many small hydrophobic molecules utilised by living organisms including steroids, vitamins, lipids, retinoids, pheromones and bilins (*Godovac-Zimmerman, 1988*). The conservation of the lipocalin family of proteins provides a transport and signalling system for these insoluble molecules in an aqueous environment. Some bind specifically

and only to particular ligands, others may be multipurpose carrier molecules,  $\beta$ LG has been shown to bind to over 100 molecules. Table 5.1 lists most of the lipocalins discovered so far, the ligand they bind to if known and their suggested function.

#### 5.1.4 Function

The lipocalins appear to cover a broad spectrum of functions. These can be roughly grouped, but much is yet to be discovered about their role and mode of action. Some lipocalins are simple carrier of vitamins or pheromones; some have enzymatic activity (prostaglandin D synthase); others exert influence on the immune system,  $\alpha_1$ -microglobulin complexes with IgA (*Åkerström & Lögdberg, 1990*), has immunosuppressive activity (*Lögdberg & Åkerström, 1981; Lögdberg et al., 1986*). Complement C8 $\gamma$  although not essential for the lytic activity of C8 in the complement pathway, enhances the binding of the C8 $\beta$  chain to C5b-7 complex.

The lipocalin family shares structural homology with another protein family that bind small hydrophobic ligands, the fatty acid-binding proteins (FABP) and with streptoavidin, a binding protein with affinity for the hydrophobic vitamin biotin. The main common structural feature all these proteins share is the folding pattern of the  $\beta$  barrel.

These proteins may provide the basis for a drug carrier system based on the structural motif of the family. The binding pocket could be modified using mutagenesis to provide a carrier molecule for a designed drug. The receptors for the majority of these molecules are as yet undiscovered but may provide the opportunity for highly specific drug delivery to particular groups of cells (*Godovac-Zimmermann, 1988*).

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Name	Species; location	Ligand	Function	References
PP14	Human endometrium	Unknown	Immunosuppression	(Seppala et al., 1994)
$\alpha_1$ -microglobulin/HC protein	Human, rat, monkey, rabbit, dolphin, pig; plasma, liver, kidney, urine, cerebrospinal fluid	yellow/brown chromophore/retinoid, porphyrin	Mediation of neutrophil chemotaxis, IgA-binding, immunosuppressive	(Åkerström & Lögdberg, 1990; Lee et al., 1987)
Retinol-binding protein	Human, rat; plasma	Retinol, retinoic acid, retinoyl acetate (narrow)	Retinol transport	(Newcomer et al., 1984; Laurent et al., 1985; Cogan et al., 1976)
$\beta$ -lactoglobulin	Cow, sheep, horse, pig, dog, dolphin; milk whey	Retinol, +100 others (broad)	Vitamin transport to the gut	(Futterman & Heller, 1972)
Insecticyanin/bilin-binding protein	tobacco hornworm, butterfly	biliverdin IX $\gamma$ (narrow)	Camouflage, photoreception?	(Holden et al., 1987; Huber et al., 1987)
Apolipoprotein D	Human plasma	cholesteryl esters, lecithin (broad)	Lipid transport	(Drayna et al., 1986)
BGprotein/Olfactory neuroepithelium protein	Frog olfactory epithelium, Bowman's gland	odorants (broad)	Odorant transfer to receptor	(Lee et al., 1987)
$\alpha$ -2 $\mu$ -globulin	Rat urine	pheromone	Sexual signalling	(Pervaiz & Brew, 1985)
Major urinary protein	Mouse urine	pheromone	Sexual signalling	(Böcskei et al., 1991)
androgen-dependent epididymal secretory protein	Rat	Retinoic acid	Sperm maturation	(Brooks et al., 1986)
Crustacyanins A & C	Lobster	Astaxanthin, retinoids	Coloration, photo-reception	(North, 1989)
Alpha-1-acid-glycoprotein	Human	progesterone (broad)	Platelet aggregation, immunosuppression	(Dente et al., 1985)
Prostaglandin D synthase	Human, Rat; brain, nervous system, retina, cochlea	prostaglandin D <sub>2</sub> , 1-chloro-2,4-dinitrobenzene, bilirubin, indocyanine green	Biosynthesis of prostaglandin D <sub>2</sub>	(Nagata et al., 1991; Urade et al., 1989)
Protein 24p3	Mouse; activated macrophages, kidney	Unknown	Defence mechanism against infection?	(Flower et al., 1991; Meheus et al., 1993)
Complement C8 $\gamma$	Human plasma	unknown/Retinol	Complement-mediated cytolysis	(Haefliger et al., 1991)
Chorioid plexus secreted protein	Toad chorioid plexus	Unknown	Transport across the blood-brain barrier?	(Achen et al., 1992)
CH21 protein/quiescence-specific polypeptide	Chicken	Unknown	Cell regulation	(Cancedda et al., 1990; Bedard et al., 1989)
Aphrodisin	Hamster	Pheromone?	Sexual signalling	(Henzel et al., 1988)
Von Ebner's gland protein	Rat	Unknown	Taste reception	(Schmale et al., 1990)
Probasin	Rat	Unknown	Unknown	(Spence et al., 1989)
Tear pre-albumin	Human tear fluid	unknown/retinol	Hydrophobic molecule carrier	(Redl et al., 1992)

**Table 5.1:** Characteristics of members of the lipocalin family

### 5.1.5 Aim of the study

The structure of PP14 was modelled using computer graphics. The purpose of this was:

1. To investigate the relationship of the structure of PP14 to that of other members of the lipocalin family, and from this determine which structural motifs are conserved in PP14 including the  $\beta$ -sheets, binding pocket and disulphide bridges. The structure obtained would also give a basis for analysing data from future X-ray crystallography experiments.
2. To analyse the binding pocket and model a potential ligands into the model of the protein, and so predict the structure of the ligand. This is particularly relevant to the activity of the recombinant protein which may require the ligand for activity.

## 5.2 METHODS

### 5.2.1 Modelling strategy

The predicted structure for PP14 was modelled using the molecular modelling programme FRODO (*Jones, 1978*) implemented on an Evans and Sutherland series 'V' workstation supporting 'crystal eyes' stereo graphics. The known X-ray structure coordinates for retinol-binding protein (RBP) and major urinary protein (MUP) were used as a framework around which to build the structure of PP14 around (*provided by Dr. SJ Yewdall*). The areas of closest sequence alignment to RBP and MUP were the framework of the polypeptide chain with the most variable sequences found in the parts joining the loop regions to the  $\beta$  strands except for specific loops with conserved sequences. The PP14 residues contributing to the framework were aligned first to the structure of MUP and, where possible, structural motifs were preserved with appropriate modifications due to sequence deletions and/or substitutions. Amino acid changes deemed of little consequence (e.g with respect to size or charge) were carried out first. Then the areas of less homology were worked on following the framework of RBP or MUP depending on which showed more homology in that particular region.

Idealised geometries, torsion (*Ramachandran & Sasisekharan, 1968*) and hydrogen-bond distances of 0.285 nm (2.85 Å) were generated throughout. Side-chains were added and their orientations chosen to give good geometry and reasonable interactions. The final model was subjected to repeated cycles of geometrically restrained regularisation and energy minimisation.

### 5.2.2 Ligand modelling

Three potential ligands were chosen to model into the proposed binding pocket of PP14: Progesterone, hyaluronic acid and prostaglandin E<sub>2</sub>. These ligands were chosen as representative of some of the groups of molecules which are present in the reproductive tract. The ligands were positioned in the molecule by eye and adjusted to give the best

orientation. The resulting models were then subjected to repeated cycles of energy minimisation to reduce conflicting interactions.

## 5.3 RESULTS

### 5.3.1 Structural features of PP14

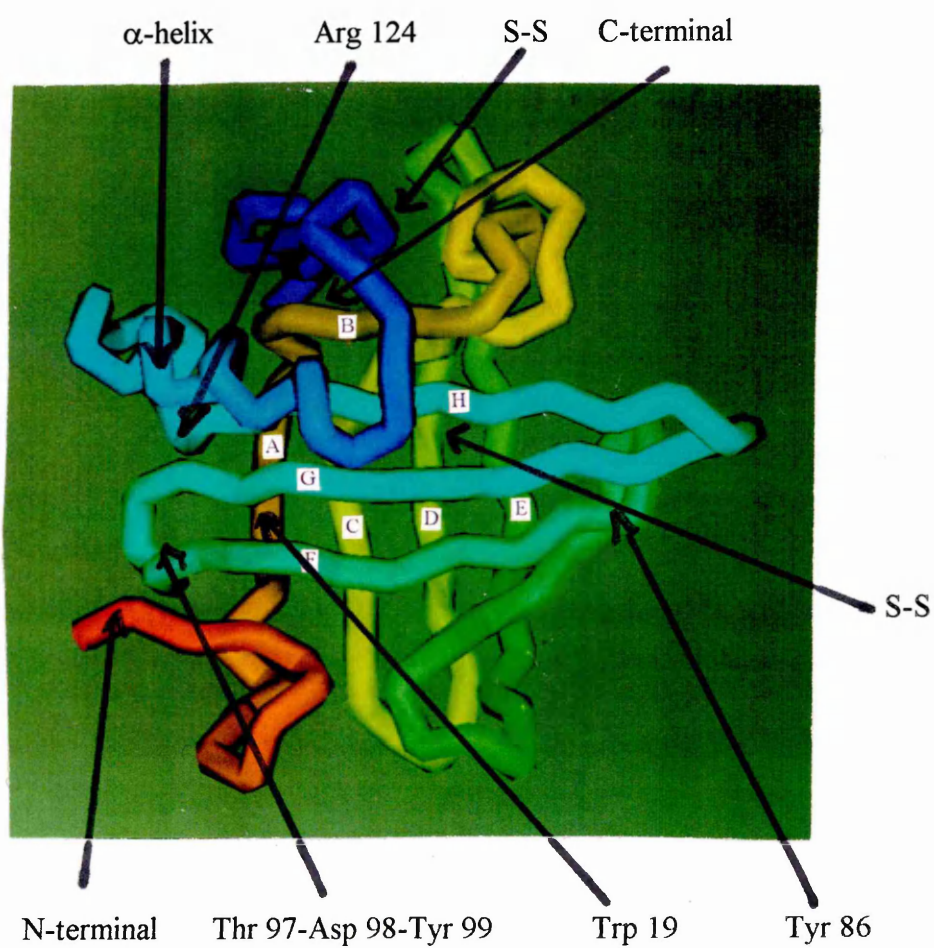
Figures 5.1 and 5.2 show the modelled predicted structure for PP14. The rainbow ribbon is coloured from red at the N-terminal to dark blue at the C-terminal. Both figures show the molecule in the same orientation to the horizontal plane but Figure 5.2 has been rotated 90° from Figure 5.1 around a vertical axis through the centre of the molecule. This means that the same features are at the top of each of the pictures, the  $\alpha$ -helix and the carboxy terminus, likewise the N-terminal is at the bottom of both pictures. The sharp bend shown in turquoise on the right of Figure 5.1 is pointing directly out of the picture in Figure 5.2.

These figures show that the predicted structure for PP14 retains many of the features common to the lipocalin family. The eight anti-parallel  $\beta$ -sheets stacked on top of each other forming a calyx are present (labelled A-H from the N-terminal end), seven of the strands cross the width of the molecule, while the second strand (B) shown in yellow is truncated. The  $\alpha$ -helix near the carboxy end is also conserved.

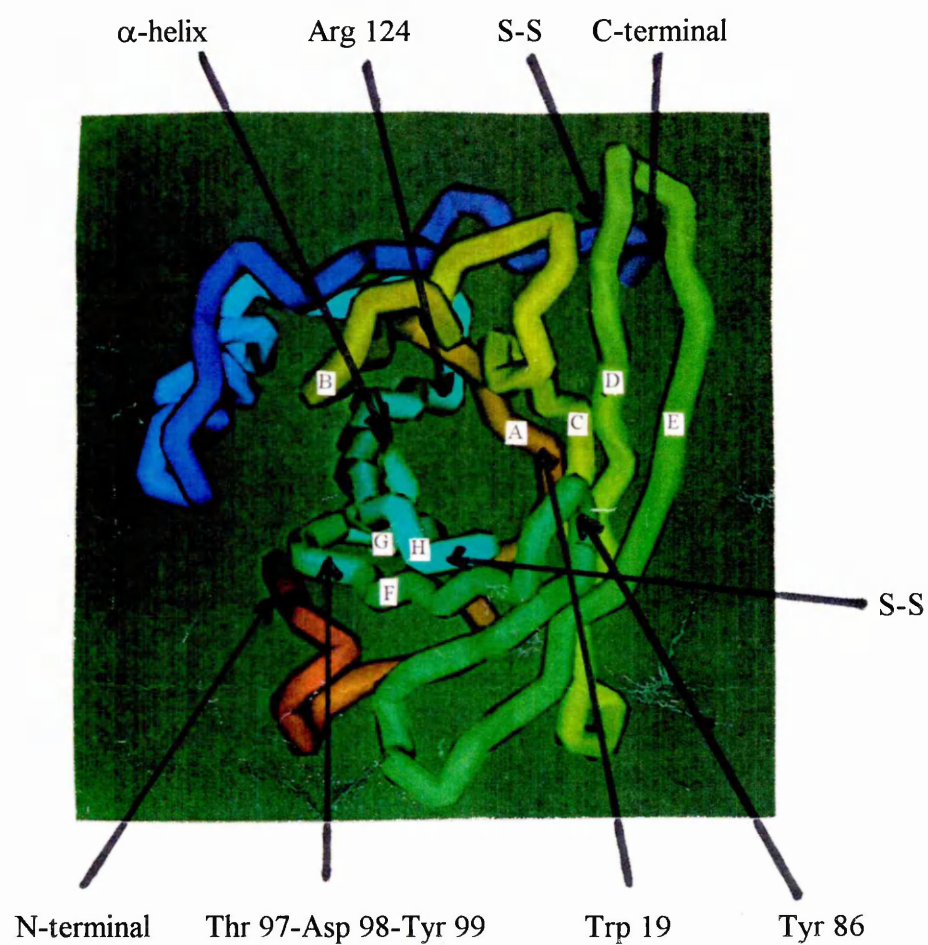
The significant residues and areas of the molecule are marked. These are the conserved triple amino acid sequence Thr-Asp-Tyr which in PP14 is at residues 97-99, the tryptophan residue at the bottom of the binding pocket which is part of a conserved triplet Gly-X-Trp, in PP14 residues 17-19 with tryptophan at residue 19, the tyrosine at the entrance to the pocket at residue 86 and the arginine behind the tryptophan at position 124. In this predicted model the loop containing the conserved triplet from 97-99 crosses the tryptophan 19 residue giving an area of the molecule with a high degree of conservation.

There are two disulphide bridges. One is shared with other lipocalins and links the C-terminal region beyond the  $\alpha$ -helix to the strand labelled E in the Figures. This is shown





**Figure 5.1:** Rainbow model of the modelled proposed structure of PP14. The N-terminal is in red and the C-terminal in blue. Significant areas of the molecule are indicated, further details are given in the text.



**Figure 5.2:** Rainbow model of the modelled proposed structure of PP14. This figure is identical to Figure 5.1 but rotated 90° around a vertical axis through the centre of the molecule. The N-terminal is in red and the C-terminal in blue. Significant areas of the molecule are indicated, further details are given in the text.

best on Figure 5.2. The other disulphide bridge links the strands labelled G and H and appears to stabilise the loop at the entrance to the binding pocket.

Figure 5.3 lists the sequence of PP14 with the significant residues marked to assist in interpreting the modelled structure.

Figure 5.4 shows the modelled structure of PP14 with the helical regions as cylinders in pink and the  $\beta$ -sheets as blue arrows. The structure of  $\beta$ -lactoglobulin is shown below in figure 5.5 for comparison. It can be seen from the two Figures that the folding of the proteins is similar and the pattern of  $\beta$ -sheets and the  $\alpha$ -helix at the C-terminal end are present in both molecules.

Figure 5.6 shows the ribbon structure from Figure 5.1 superimposed on the modelled position of the outline of the amino acid structures with progesterone modelled into the binding pocket to demonstrate the orientation of the pocket within the molecule. The electron densities surrounding the ligand are also shown.

### **5.3.2 Binding pocket**

An analysis of the residues lining the binding pocket shows them to be predominately hydrophobic consisting of leucine, valine, alanine and isoleucine. The exceptions to this are Glu 55 and Asp 109 which are negatively charged and Asn 90. The dimensions of the binding pocket measured from the model are 12.8Å by 9.5Å by 5.6Å, pinched in the middle to 4.7Å.

### **5.3.3 Ligand binding**

The structures of the three ligands modelled into the binding pocket are shown in Figure 5.7, 5.8 and 5.9. From these structures it can be seen that progesterone has two polar oxygen groups, one at either end, shown in red (Figure 5.7). Hyaluronic acid has one nitrogen (shown in blue) and several oxygen groups (shown in red) distributed across the

molecule (Figure 5.8). Prostaglandin E<sub>2</sub> has oxygens at either end and one in the middle of the molecule (Figure 5.9). It is important to consider the position of these polar groups in relation to the groups within the binding pocket of the molecule with which they may interact.

Figures 5.10, 5.11 and 5.12 show progesterone modelled into the binding pocket from three different angles. In Figures 5.10 and 5.11 the orientation of the model of PP14 is the same as shown in Figure 5.1, with Trp 19 on the left and Tyr 86 on the right marked on the Figures. Figure 5.12 shows progesterone from the angle looking down into the binding pocket. There is some overlapping of the Van derWaal shells of the PP14 model and progesterone in these Figures. This is due to amino acids in front or behind the molecule overlapping in the pictures. However it can be seen from the spacing of the amino acid side chains and Figure 5.12 that progesterone fits into the pocket.

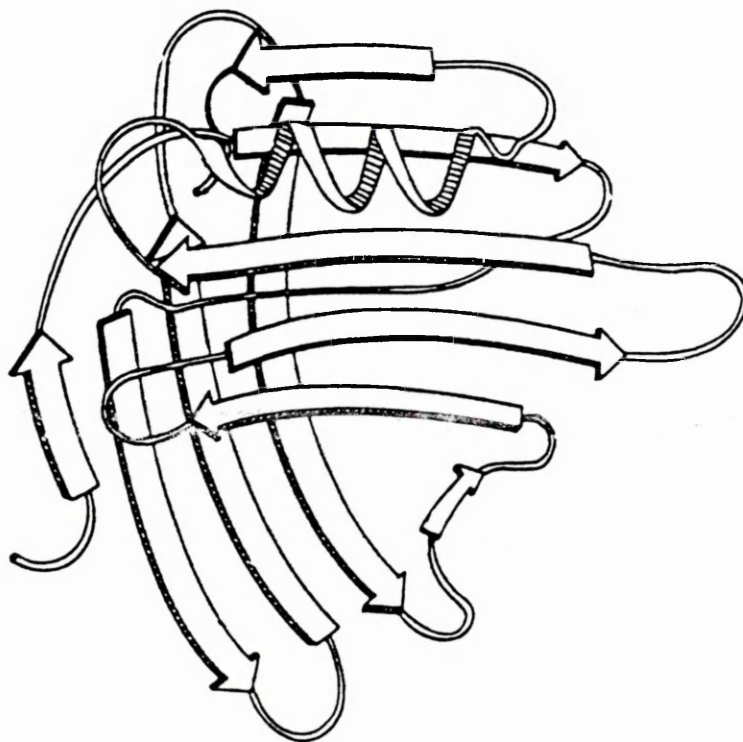
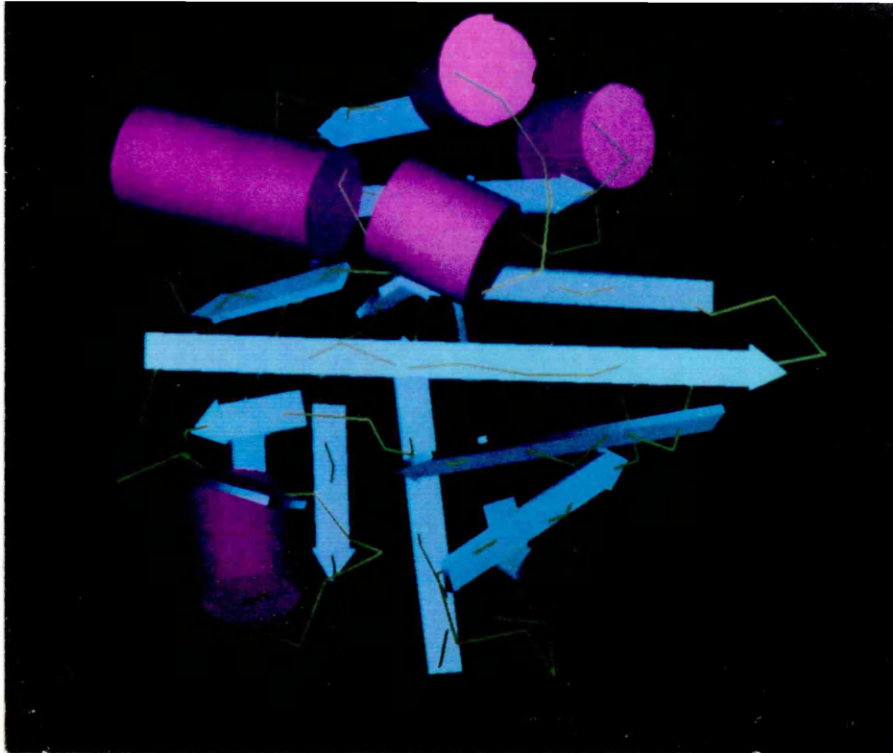
Figures 5.13 and 5.14 demonstrates the orientation of the progesterone molecule within the ribbon diagrams of the PP14 model. This shows the oxygen groups (shown in red) of the progesterone molecule and their position. Also marked on this diagram are the three amino acids lining the binding pocket which may interact with these oxygen molecules, Glu 55, Asn 90 and Asp 109. From this Figure it can be seen that these residues are all in the same regions as the oxygen molecules.

Figures 5.15 and 5.16 show hyaluronic acid modelled into the binding pocket. Although this molecule fitted well from a size and shape perspective, the charged groups in the centre of the molecule caused clashing interactions with the hydrophobic amino acids in these regions.

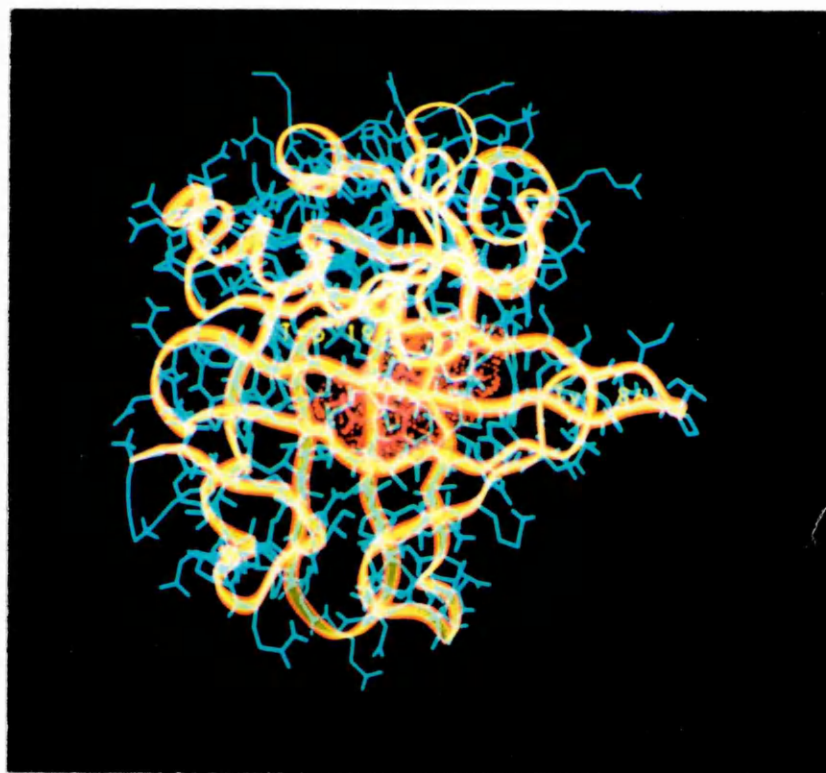
Prostaglandin E<sub>2</sub> was too large to fit in the pocket. Although it looks a similar size to the other two molecules it's double side chain meant that the interactions with the amino acids lining the pocket were too close.

1 Met	2 Asp	3 Ile	4 Pro	5 Gln	6 Thr *	7 Lys *	8 Gln	9 Asp	10 Leu	11 Glu	12 Leu	13 Pro	14 Lys A *	15 Leu A	16 Ala A
17 Gly A *	18 Thr A *	19 Trp A *	20 His A *	21 Ser A	22 Met A *	23 Ala A *	24 Met	25 Ala B *	26 Thr B	27 Asn B	28 Asn B	29 Ile	30 Ser	31 Leu *	32 Met
33 Ala	34 Thr	35 Leu	36 Lys	37 Ala *	38 Pro	39 Leu	40 Arg *	41 Val *	42 His	43 Ile	44 Thr C *	45 Ser C	46 <i>Leu</i> C	47 <i>Leu</i> C	48 Pro C
49 Thr C	50 Pro	51 Glu D *	52 Asp D	53 Asn D	54 <i>Leu</i> D *	55 <i>Glu</i> D	56 <i>Ile</i> D	57 <i>Val</i> D	58 <i>Leu</i> D	59 His D *	60 Arg	61 Trp E	62 Glu E	63 Asn E *	64 Asn E
65 Ser E	66 Cys E *	67 <i>Val</i> E	68 Glu E *	69 Lys E	70 Lys E	71 <i>Val</i> E	72 <i>Leu</i>	73 Gly	74 Glu	75 Lys *	76 Thr *	77 Gly	78 Asn	79 Pro *	80 Lys
81 Lys *	82 Phe *	83 Lys *	84 Ile	85 Asn	86 Tyr *	87 Thr	88 <i>Val</i> F	89 <i>Ala</i> F	90 <i>Asn</i> F *	91 Glu F	92 <i>Ala</i> F	93 Thr F *	94 <i>Leu</i> F	95 <i>Leu</i> F	96 Asp F
97 Thr F *	98 Asp F *	99 Tyr F *	100 Asp	101 Asn	102 Phe G *	103 <i>Leu</i> G *	104 Phe G	105 <i>Leu</i> G	106 Cys G *	107 <i>Leu</i> G *	108 Gln G	109 <i>Asp</i> G	110 Thr G	111 Thr G	112 Thr G
113 Pro	114 Ile H	115 Gln H	116 Ser H	117 Met H	118 Met H	119 Cys H	120 Glu H	121 Tyr H	122 Leu H	123 Ala H	124 Arg *	125 Val $\alpha$	126 Leu $\alpha$	127 Val $\alpha$	128 Glu $\alpha$
129 Asp $\alpha$	130 Asp $\alpha$	131 Glu $\alpha$	132 Ile $\alpha$ *	133 Met $\alpha$	134 Gln $\alpha$ *	135 Gly $\alpha$	136 Phe $\alpha$ *	137 Ile $\alpha$	138 Arg $\alpha$ *	139 Ala $\alpha$	140 Phe $\alpha$	141 Arg $\alpha$	142 Pro $\alpha$	143 Leu $\alpha$	144 Pro $\alpha$
145 Arg $\alpha$	146 His	147 Leu *	148 Trp	149 Tyr	150 Leu	151 Leu	152 Asp	153 Leu *	154 Lys	155 Gln	156 Met	157 Glu	158 Glu	159 Pro	160 Cys *
161 Arg	162 Phe														

**Figure 5.3:** Amino acid sequence of PP14. The significant residues are shown in bold, the regions forming the strands and  $\alpha$ -helix are labelled A-H and  $\alpha$ , the residues mentioned in the text: Trp 19, Tyr 86, Arg 124 are shaded lightly and the cysteine residues are shaded heavily. The residues lining the binding pocket are in italics and the residues which have homology with MUP or RBP are indicated by \*

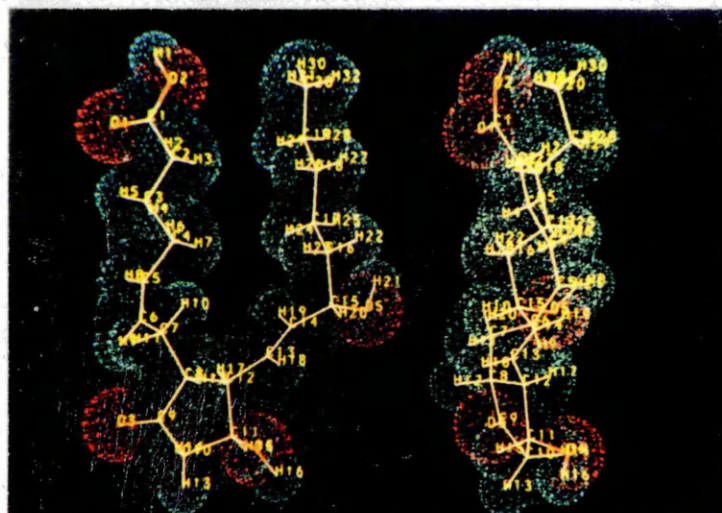
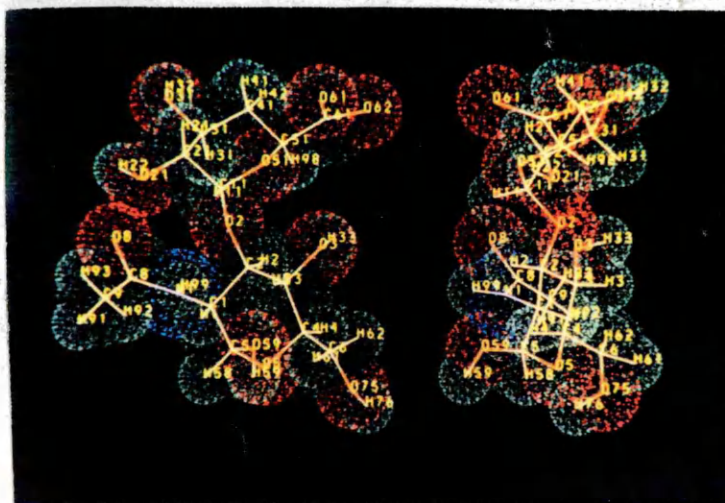
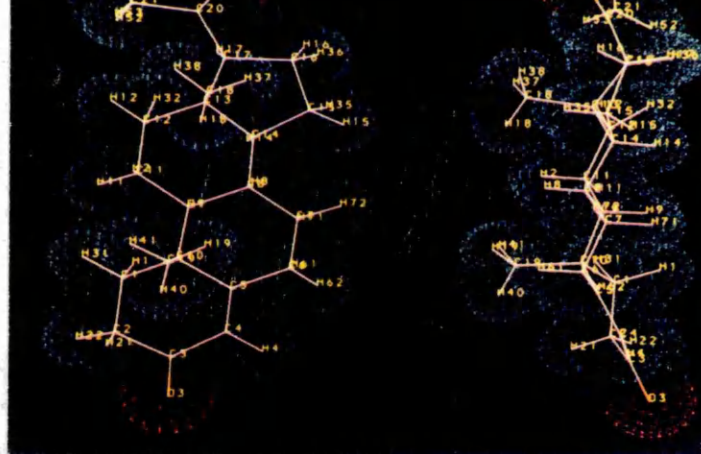


**Figure 5.4:** The structure of PP14 with  $\alpha$ -helical regions shown as barrels in pink,  $\beta$ -sheets are shown as arrows in blue. The structure of  $\beta$ -lactoglobulin (*Nagata et al., 1991*) is shown below in **Figure 5.5** for comparison of the two structures.



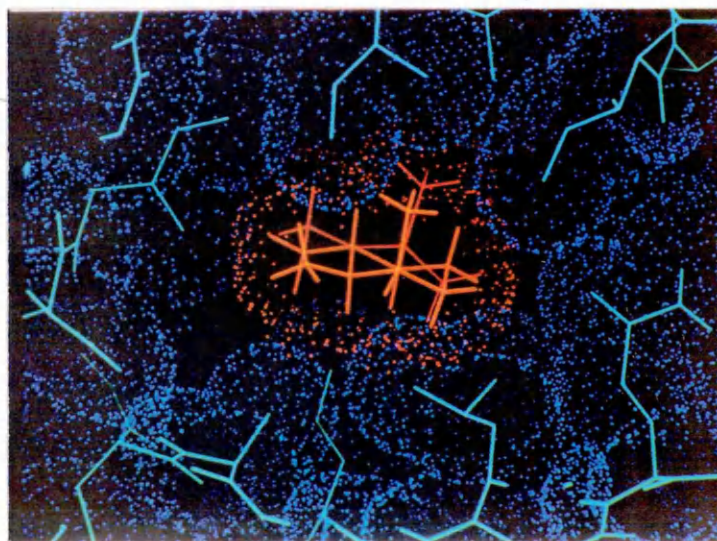
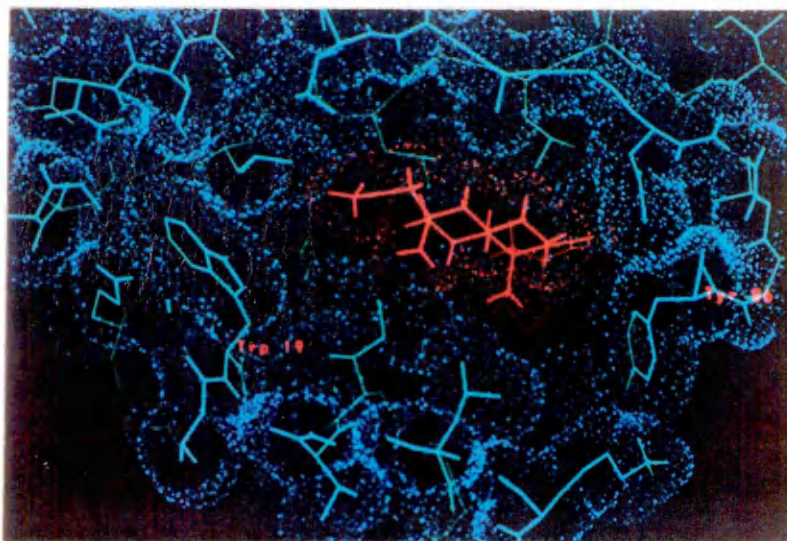
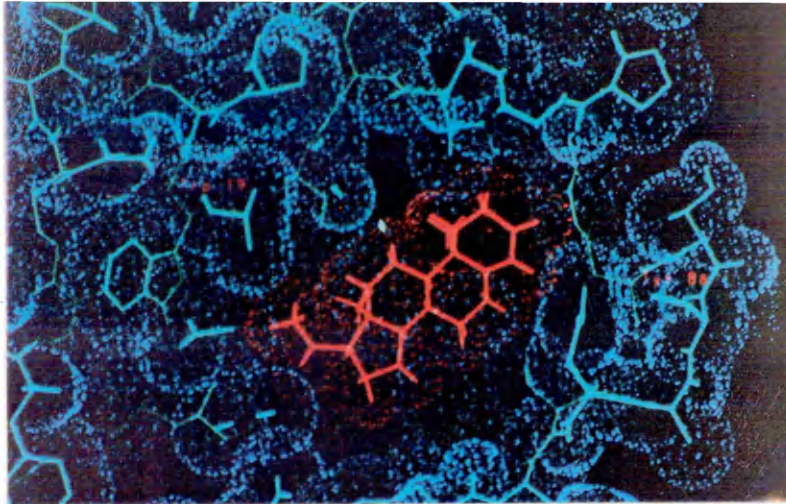
**Figure 5.6:** The ribbon model of PP14 super-imposed over the amino acid structure. Progesterone is shown in the binding pocket to show the size and position of the pocket.



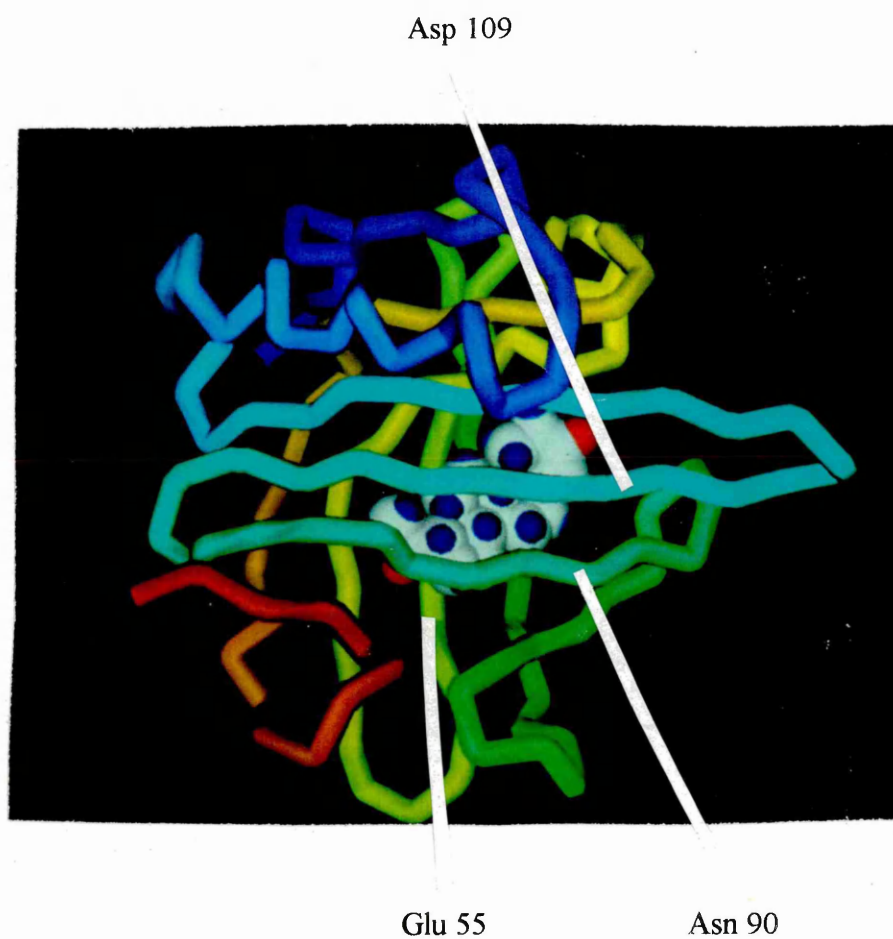


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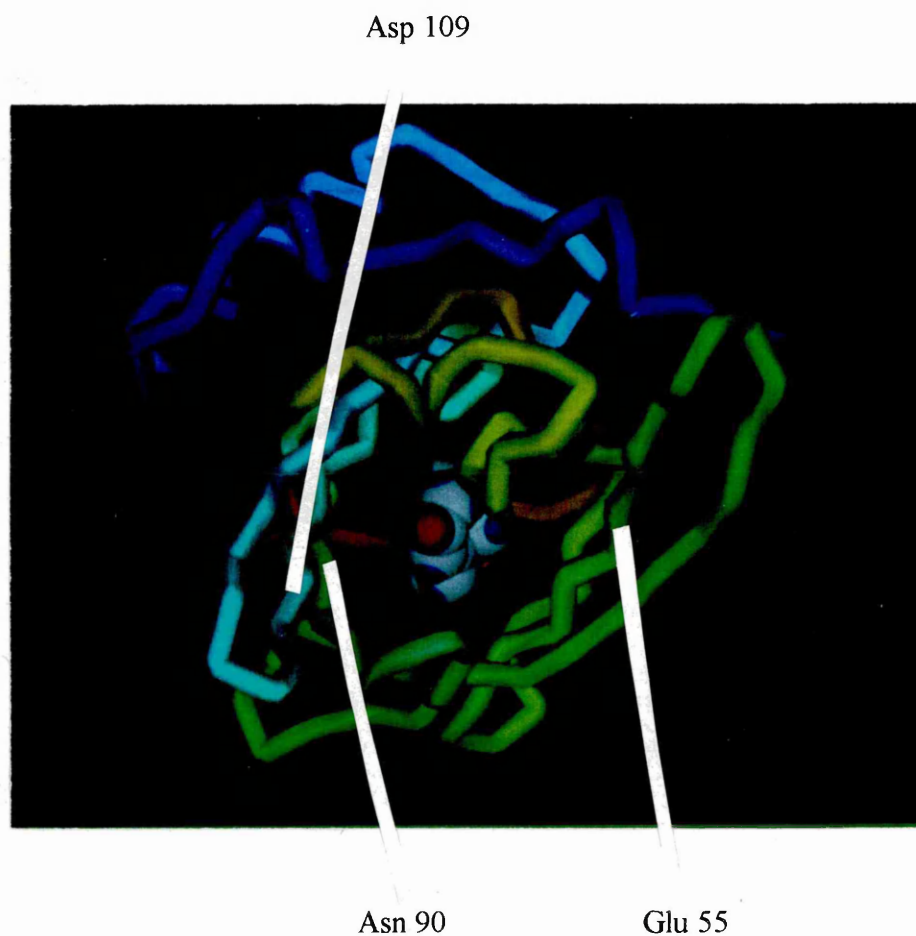




**Figures 5.10, 5.11, 5.12:** Progesterone modelled into the binding pocket of PP14 shown from three different angles.

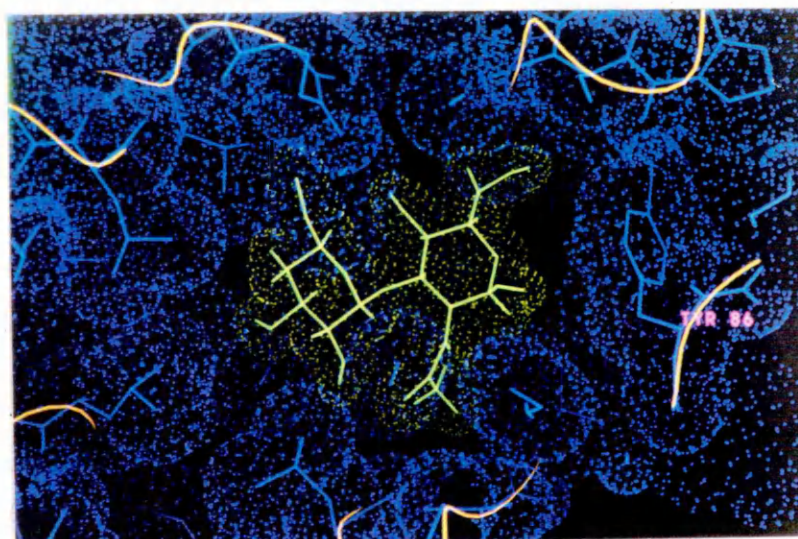
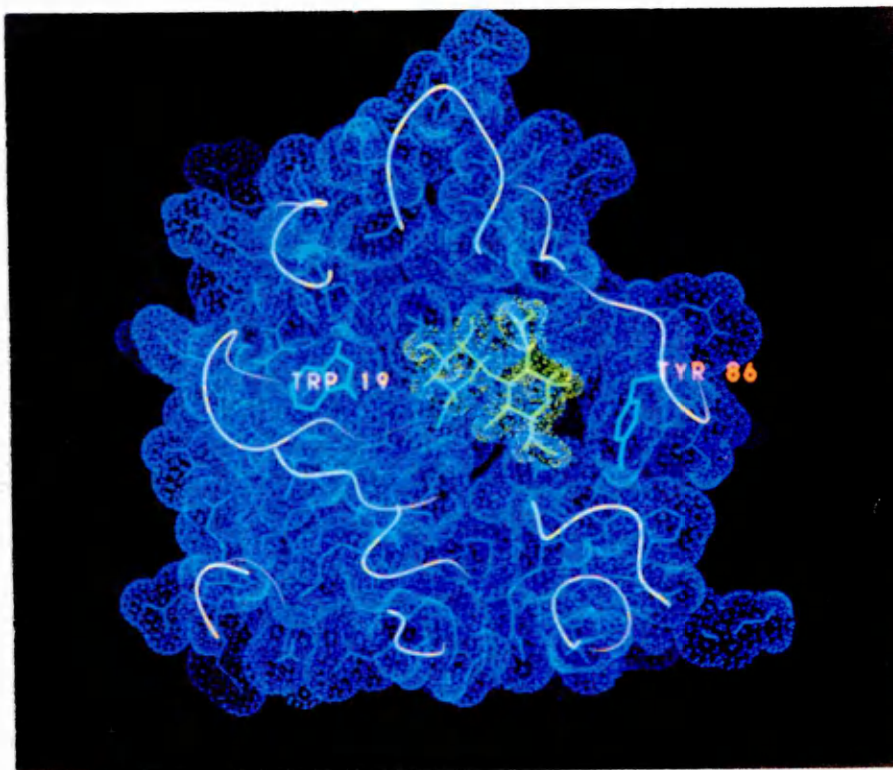


**Figure 5.13:** Progesterone modelled into the rainbow ribbon model of PP14 showing the orientation of the charged groups in the progesterone structure and the possible interactions with Glu 55, Asn 90 and Asp 109 in the binding pocket of PP14.



**Figures 5.14:** Progesterone modelled into the rainbow ribbon model of PP14, looking into the binding pocket, showing the orientation of the charged groups in the progesterone structure and the possible interactions with Glu 55, Asn 90 and Asp 109 in the binding pocket of PP14.





**Figures 5.15 and 5.16:** Hyaluronic acid modelled into the binding pocket of PP14.

## 5.4 DISCUSSION

Molecular modelling is a technique used in conjunction with X-ray crystallography to elucidate the molecular structure of proteins. X-ray crystallography is required to definitively determine the crystalline structure of a protein. However much useful information can be gained from predicting the structure of a protein by aligning the amino acid sequence with that of another protein where homology of sequence exists and structural homology is anticipated. X-ray crystallography is a time-consuming and lengthy technique. In the case of PP14 it was anticipated that the recombinant protein might require a co-factor or ligand to be active, and that studies on this activity would be held up due to a lack of information on possible ligands.

Therefore, in view of the known structural homology between other members of the lipocalin family, molecular modelling by the alignment of amino acid sequences was possible, and used as a tool to predict the molecular structure of PP14. From this it was hoped that information would be gained about the binding pocket, in order to predict likely ligands. X-ray crystallography studies are currently underway to complement the work reported here.

The results of the molecular modelling of PP14 show that, from the proposed model, PP14 is predicted to exhibit the typical structural features of the lipocalin family. Some aspects of the model merit particular mention. The  $\beta$ -sheet folding pattern common to all the lipocalins is present and the predicted secondary structure shows similarity with the structure of beta-lactoglobulin. The  $\alpha$ -helix at the carboxyl end is present with the disulphide bridge linking region beyond the helix into the rest of the molecule. There is also a second disulphide bond linking the 6th and 7th  $\beta$ -sheets. This bond is found in a few of the other lipocalins, but is not a strongly conserved feature.

The Trp residue which is conserved in all lipocalins so far identified is at position 19 in PP14. This residue in this model is positioned at the bottom of the binding pocket, on

the first  $\beta$ -strand. The regions on either side show some partial homology with other members of the family. This area is crossed by a loop formed of residues that also share homology with other lipocalins.

Of particular interest is residue 86, a tyrosine residue which is directly adjacent to the opening and has freedom of movement. This suggests that it could pivot to close the binding pocket after the ligand has entered. This closing action may only be possible after a conformational shift when the ligand has been bound and the tyrosine residue is then able to move to a different conformation to complete the binding site at the opening of the pocket. The second disulphide bridge which is not common to all lipocalins stabilises the  $\beta$ -sheets which interact with this Tyr residue, indicating that the conformation of this part of the molecule may be important.

The binding pocket is extremely hydrophobic, all the amino acid residues lining it are hydrophobic with the exception of Glu 55, Asn 90 and Asp 109. These residues may interact with charged groups on the ligand. From the proposed structure the pocket is elongated, pinched in the middle, with a bulge at each end. .

Three ligands were modelled into the pocket. Progesterone and prostaglandin  $E_2$  both have immunosuppressive activity and are found in the endometrium. Hyaluronic acid may bind to adhesion molecules that are involved in the interaction between the trophoblast and the decidua during pregnancy, and has a two ring structure that may fit the bulged sections of the binding pocket.

Of the three ligands, the best fit was progesterone, due to its size and shape and the positioning of its charged groups. However a systematic analysis of a range of ligands would need to be carried out before it could be claimed that the ligand for PP14 is in fact progesterone.

Hyaluronic acid, while fitting well into the pocket from a structural point of view, has many charged groups which did not match the predominately hydrophobic environment of the binding pocket.

Prostaglandin E<sub>2</sub> did not fit into the pocket due to the two side chains that are part of the structure of this molecule.

These results predict that the ligand for PP14 is a hydrophobic molecule with a polar or charged group on either end. The structure of the ligand may be in two parts linked by a narrower part, to fit the bulges and pinched middle section of the binding pocket of PP14. Progesterone comes close to fulfilling these criteria, and may be the ligand, but many other molecules could also fit this description.

The activity of PP14 is likely to be associated with either the section of the molecule at the back of the binding pocket, or the section around the entrance to the pocket. The model predicted here could be used to design peptides from these parts of the molecule which could mimic the activity of PP14.

## 5.5 CONCLUSION

The predicted molecular structure for PP14, a lipocalin, has been proposed. This modelled structure demonstrates that this molecule has a binding pocket and shares areas of conservation with other members of the lipocalin family. The ligand for PP14 is still unknown, although progesterone fits into the pocket there is not yet enough evidence to claim that it is the ligand for PP14.

This structural information will help in further work to discover the co-factor or ligand that PP14 binds. This is particularly relevant to the expression of PP14 as an active recombinant protein. The model also provides information that may be useful in the investigation of the mode of action of the molecule.



## CHAPTER SIX: CLINICAL STUDIES

### 6.1 INTRODUCTION

Placental protein 14 (PP14) is the most abundant protein product released from epithelial cells into the lumen of the endometrium in the secretory phase of the menstrual cycle and during the first trimester of pregnancy (*Olajide & Chard, 1992*). Although the exact role of this protein has yet to be elucidated it may be involved in the regulation of the maternal immune system to protect the fetus from adverse immune responses (*Bolton et al., 1987*) or play a part in paracrine interactions between the endometrium and trophoblast (*Seppälä et al., 1994*).

The traditional way to study the development of the endometrium is by taking an endometrial biopsy (*Noyes et al., 1950*). This involves the removal of a small sample of endometrial tissue from the patient and the subsequent histological examination of the specimen. This method has significant disadvantages. The first and probably most important is that it is an uncomfortable and often painful procedure for the patient, particularly if she is nulliparous (*Li et al., 1990*), there is also a risk of disrupting an early pregnancy (*Li et al., 1993a*). The histological examination of the tissue is a subjective evaluation, requiring an experienced observer, although recently semi-quantitative methods have been described using morphometric analysis (*Li et al., 1988*), and therefore a more objective measure of endometrial status may be obtained. In addition the histological examination of a biopsy gives no information about the functional secretory status of the endometrium. Immunohistochemical and in situ hybridisation techniques can be carried out on biopsy specimens yielding further information about the presence of proteins within the tissue. These techniques, however, give no information about proteins secreted into the endometrial environment. Because of these problems, endeavours have been made to develop alternative non-invasive methods of studying the endometrium. There is some evidence to support the theory that the main abnormality in the endometrium of infertile subjects involves the secretory activity of the glandular

epithelium. In this respect PP14 levels in plasma have been measured by a number of investigators in a wide range of women; (*Joshi et al., 1986; Julkunen et al., 1986b*). However plasma PP14 concentrations may not accurately reflect the situation in the endometrium (*Wood et al., 1990*) and many studies have found no significant correlation between plasma PP14 and endometrial morphology (*Than et al., 1988; Edwards, 1988*).

PP14 secreted from the glandular endometrium can be recovered from uterine luminal fluid (*Bell & Doré-Green, 1987; Bell, 1988*). Sampling this fluid or flushing out the uterine cavity provides the opportunity for a more direct measurement of the secretions of the endometrium, and therefore its functional status. This study investigates the potential of the technique of uterine flushing with particular application to the study of PP14 concentrations in fertile women, infertile women and women with recurrent miscarriage. It also compares results from the techniques of uterine flushing and endometrial biopsy in the recurrent miscarriage patients.

#### **6.1.1 Collaborators**

This study was part of a collaboration with Mr. T.C. Li from the Jessop Hospital for Women, Sheffield and his clinical and laboratory staff teams. The technique of uterine flushing was developed by Mr. Li as part of the study and the uterine flushings, endometrial biopsies and plasma samples were obtained by the clinical staff at the hospital. The biopsy samples were processed by staff at the Department of Biomedical Sciences, University of Sheffield, and analysed by Mr. Li..

#### **6.1.2 Purpose of the study**

1. To establish the levels of PP14 present in uterine flushings from fertile women throughout the menstrual cycle.
2. To compare the levels of PP14 measured in uterine flushings from fertile women with those measured in plasma samples taken immediately afterwards.
3. To compare the levels of PP14 in uterine flushings and plasma from patients with unexplained infertility with the levels found in fertile women.

4. To compare the levels of PP14 in uterine flushings and plasma from patients with recurrent miscarriage with the levels found in fertile women.
5. To compare the levels of PP14 in uterine flushings from women with recurrent miscarriage with the dating of their endometrial biopsies and so establish if PP14 levels measured in uterine flushings have a diagnostic value in the study of endometrial function.
6. To investigate whether the PP14 levels had any value in predicting the success of recurrent miscarriage patients in their next attempted pregnancy.

## **6.2 SUBJECTS AND METHODS**

### **6.2.1 Ethical aspects**

Informed consent was obtained from all subjects participating in this study. Approval for the study was obtained from the local Ethics Committee.

### **6.2.2 Fertile subjects**

Twenty-three healthy fertile women were recruited as a control group from the gynaecological outpatient clinic, Jessop Hospital for Women. To be included in the study the women had to fulfill the following criteria:

1. Aged between 20 and 40 years (range 24-40, median = 32 years).
2. Regular cycles between 25 and 35 days.
3. Previously fertile (parity ranged from 1 to 4, median = 2)
4. Had not taken any steroid hormones in the two months before the study.
5. No known history or evidence of uterine pathology.

Twenty of the women had been previously sterilised by tubal ligation (87%), the remaining 3 (13%) were using the condom as contraception during the study.

### **6.2.3 Infertile subjects**

Eighteen women with unexplained infertility were recruited from the infertility outpatient clinic, Jessop Hospital for Women. The patients were defined as having unexplained infertility if they had been trying to conceive for more than 18 months without success, and without a cause for this lack of fertility being discovered. The range of duration of infertility was 3 to 10 years (median 5 years). The women all had regular menstrual cycles ranging from 25 to 35 days, and evidence of ovulation from one or two plasma progesterone levels over 5.8ng/ml (18nmol/l) in the luteal phase of the cycle (*WHO, 1984*). They all had normal thyroid function tests, normal plasma prolactin

concentrations, normal hysterosalpingogram and normal tubes by laparoscopic investigation. All the partners of the women had previously undergone semen analysis. The results of these analyses were compared with the parameters measured in semen analyses taken from a group of fertile men from the local population of Sheffield, as recommended by Dunphy et al., (1991). Any couples where the male partner's semen was defined as abnormal based on this comparison were excluded from the study.

#### **6.2.4 Recurrent miscarriage subjects**

35 women were recruited from the recurrent miscarriage clinic, Jessop Hospital for Women. Recurrent miscarriage was defined as three or more consecutive pregnancy losses with normal karyotype before 20 weeks gestation. 25 of the women were primary miscarriage patients (nulliparous) and 10 were secondary miscarriers (at least one live birth,  $av=1$ ).

#### **6.2.5 Timing of sample collection**

All the women collected early morning plasma or urine samples from day nine of their cycle counting from the first day of bleeding of their last menstrual period. Luteinising hormone (LH) was measured in these specimens by immunoradiometric assay (*Li et al., 1992*) and the day on which the LH surge occurred was designated as day LH+0. The LH surge was defined as a rapid rise in plasma LH to a peak of  $> 20\text{iu/l}$  followed by a fall to basal plasma level within 3 days. All plasma samples and uterine flushings were timed according to the LH surge.

#### **6.2.6 Uterine flushings**

Uterine flushings were carried out as an out-patient procedure. A bivalve speculum was inserted in the vagina and the cervix exposed and cleaned with physiological saline. A size eight Foley catheter was passed through the the cervix into the uterine cavity and the balloon was inflated with 1.5ml of physiological saline. The cavity was gradually flushed twice with 2ml of physiological saline. The same flushing procedure was repeated a further four times making a total of 10ml of saline. The fluid obtained was mixed,

pooled and stored at -20°C until assayed for PP14. In most cases several flushings were taken from each women, with a minimum interval of 48 hours between samples.

#### **6.2.7 Endometrial biopsy samples**

Endometrial biopsies were taken from the recurrent miscarriage patients. All the biopsies were taken on day LH +7 of the cycle, immediately after the uterine flushing. The biopsy was taken using a Sharman's curette from the fundus and upper part of the body of the uterus. The specimens were fixed in 2% glutaraldehyde in sodium cacodylate buffer (pH 7.4) for 4-6 hours, then washed in buffer, dehydrated and embedded in JB-4, a plastic polymer. They were then sectioned at 2µm and the sections stained with acid Fuchsin and Toluidine Blue then examined by light microscopy. All the endometrial biopsies were studied histologically and analysed by two different methods: the dating criteria of Noyes et al., (1950) and morphometric analysis (*Li et al., 1988*). Endometrial biopsy samples were considered retarded if the histological dating results were >2 days behind the chronological dating.

#### **6.2.8 Plasma samples**

Immediately after the uterine flushing and endometrial biopsy, if taken, a 10ml blood sample was taken. The samples were collected in tubes containing lithium heparin and centrifuged at 1500g for 10 minutes to obtain plasma which was stored at -20°C until subsequent PP14 assay.

#### **6.2.9 PP14 assay**

The PP14 assay was carried out as described in Appendix II. Quality controls at 50ng/ml and 10 ng/ml were included in each assay. For assays measuring the PP14 content of plasma the quality controls were made by spiking normal male plasma with PP14 purified as described in chapter 2. For assays measuring the PP14 content of uterine washings the quality controls were made up in the same way but diluted in assay buffer (phosphate buffer, pH 7.5, 2% horse serum, 0.1% sodium azide). Plasma samples and plasma quality controls were diluted 1:1 with assay buffer before measurement, this eliminated

some non-specific variation observed when neat serum was assayed. Uterine flushings were diluted with assay buffer if necessary until the sample could be measured from the standard curve. Parallelism of response has already been established for this assay with plasma and amniotic fluid (*Bolton et al., 1983*), this was found to hold true for uterine flushings diluted with assay buffer. The working range of the assay was taken as between 3 ng/ml and 100 ng/ml and the inter-assay and intra-assay coefficients of variation (CV) were less than 10%. The detection limit of the assay was 3ng/ml. Each sample was assayed in duplicate at least twice and the average of the measurements taken.. Assays where the quality controls indicated that the inter-assay CV was more than 10% were excluded, likewise individual sample measurements with an intra assay CV of more than 10% were discarded.

#### **6.2.10 Statistical analysis**

Where four or more samples were measured on the same LH day the geometric mean was calculated. The uterine flushing and plasma PP14 results from one infertile patient were more than two standard deviations outside the mean. Since this result may indicate some underlying pathology the results from this patient were excluded from the calculation of the mean. All the original data points are shown on each graph, the summary graphs show the adjusted data.

The geometric means from the results from fertile women were compared with those from infertile women and from women suffering from recurrent miscarriage using Student's *t*-test. The level of statistical significance was chosen at  $P < 0.05$ .

The correlation between levels of PP14 measured in uterine flushings and in plasma samples was analysed using regression analysis.

## 6.3 RESULTS

### 6.3.1 PP14 levels in uterine flushings and plasma samples from fertile women

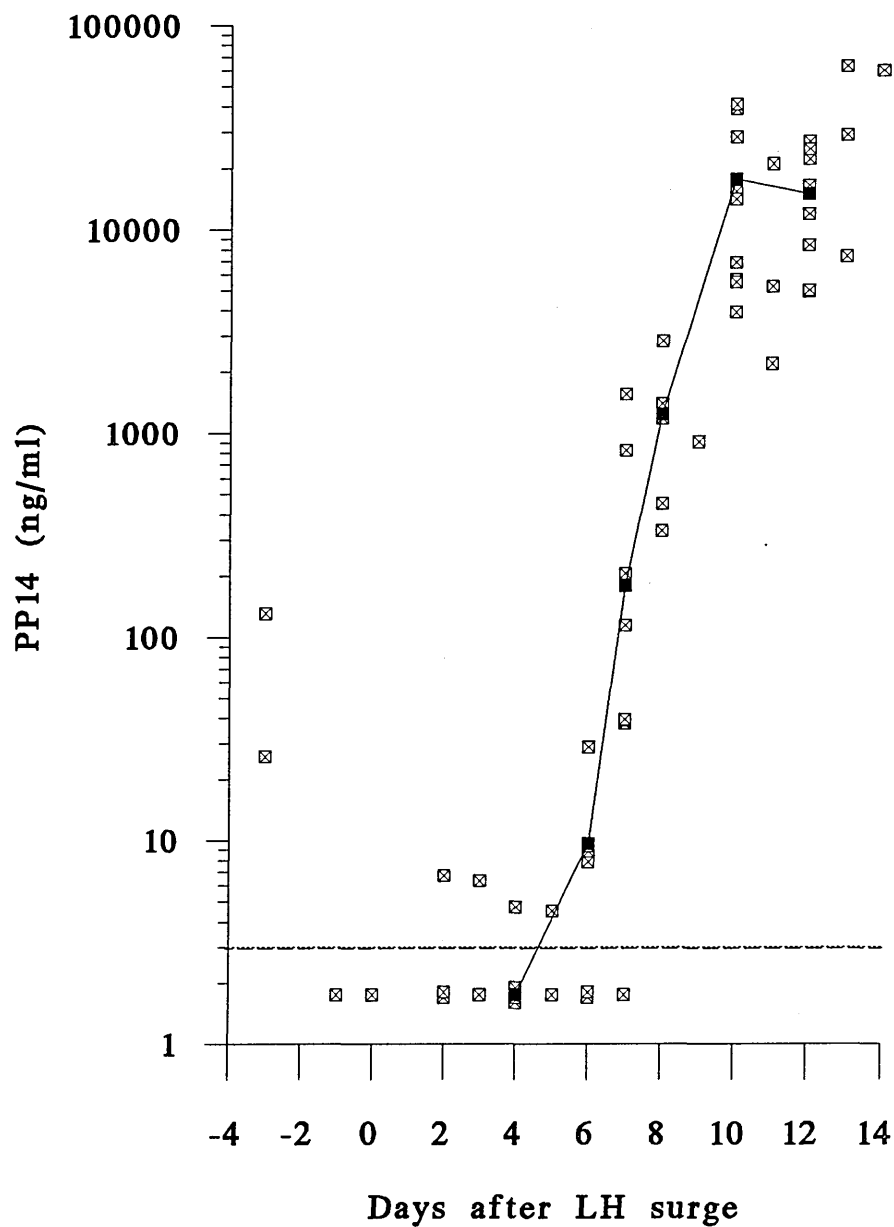
A total of 60 uterine flushings were carried out on fertile women and 53 plasma samples taken between days LH -3 and LH +14 of the menstrual cycle.

The concentrations of PP14 measured in uterine flushings from fertile women are shown in Figure 6.1. PP14 was detectable in two samples taken on day LH -3. Between day LH -1 and LH + 5 the levels of PP14 measured were mostly below or just above the detection limit of the assay. After day LH +6 the PP14 levels started to rise rapidly. The mean PP14 concentrations in uterine flushings were: day LH +4 = not detectable, LH +6 = 9.6 ng/ml, LH +7 = 178.9 ng/ml, LH +8 = 1228.1 ng/ml, LH +10 = 17529.1 ng/ml, LH +12 = 14861.5 ng/ml.

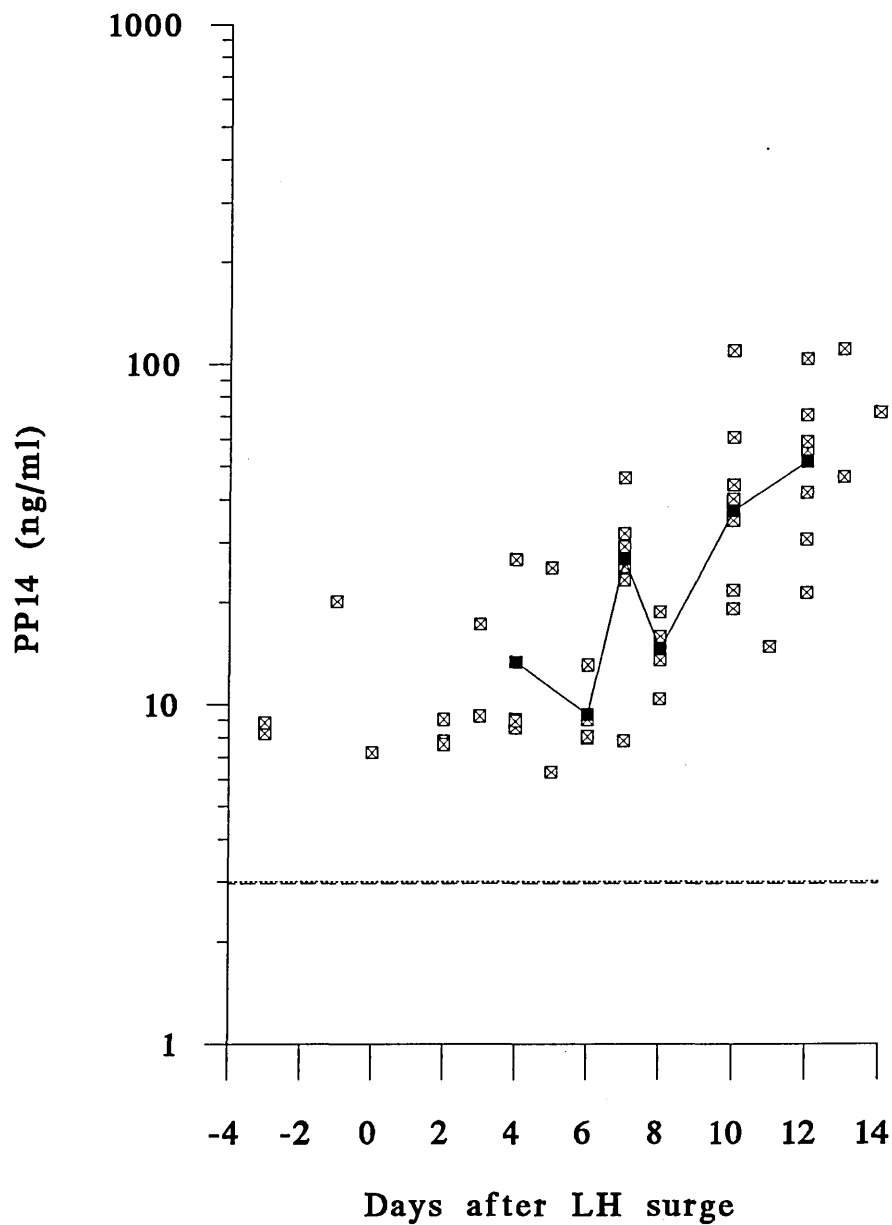
The concentrations of PP14 measured in the plasma samples from fertile women are shown in Figure 6.2. PP14 was detectable in all plasma samples and the levels rose during the secretory phase of the menstrual cycle although there was a small drop at day LH +8. The mean concentrations in the plasma samples were: day LH +4 = 13.3 ng/ml, LH +6 = 9.3 ng/ml, LH +7 = 26.8 ng/ml, LH +8 = 14.5 ng/ml, LH +10 = 36.9 ng/ml, LH +12 = 51.4 ng/ml.

The correlation between levels of PP14 in uterine flushings and plasma samples taken immediately afterwards is shown in figure 6.3. There is a significant ( $P < 0.0005$ ) correlation between PP14 levels measured in the uterine flushings and plasma samples ( $r = 0.64$ ;  $n = 53$ ). The concentration of PP14 in uterine flushings rose more rapidly than that measured in plasma samples although both rose during the menstrual cycle to reach a peak in the late secretory phase. At this stage the levels of PP14 detected in uterine flushings are over 100 times greater than those measured in plasma samples.

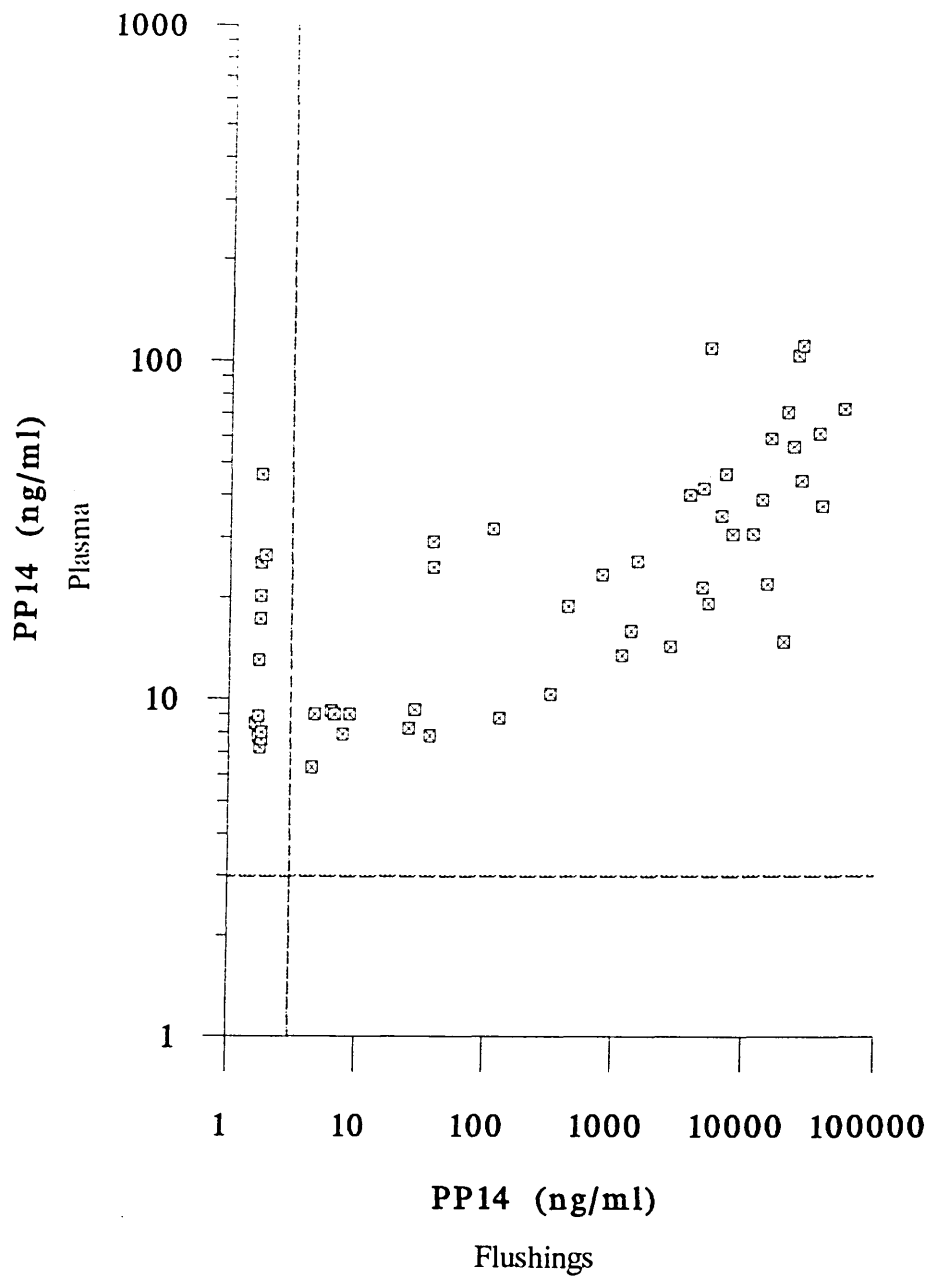




**Figure 6.1:** The concentration of PP14 in uterine flushings (n=60) obtained from 23 fertile women with regular menstrual cycles. On days where there were four or more results the means are represented by the closed squares. The dotted line indicates 3 ng/ml, the detection limit of the assay.



**Figure 6.2:** The concentration of PP14 in plasma samples (n=53) obtained from 20 fertile women with regular menstrual cycles. On days where there were four or more results the means are represented by the closed squares. The dotted line indicates 3 ng/ml, the detection limit of the assay.



**Figure 6.3:** The relationship between the concentration of PP14 in uterine washings (x-axis) and the concentration of PP14 in plasma samples (y-axis) taken immediately afterwards from fertile women ( $n=53$ ). The dotted lines indicate 3 ng/ml, the detection limit of the assay. ( $r=0.64$ ,  $P<0.0005$ )

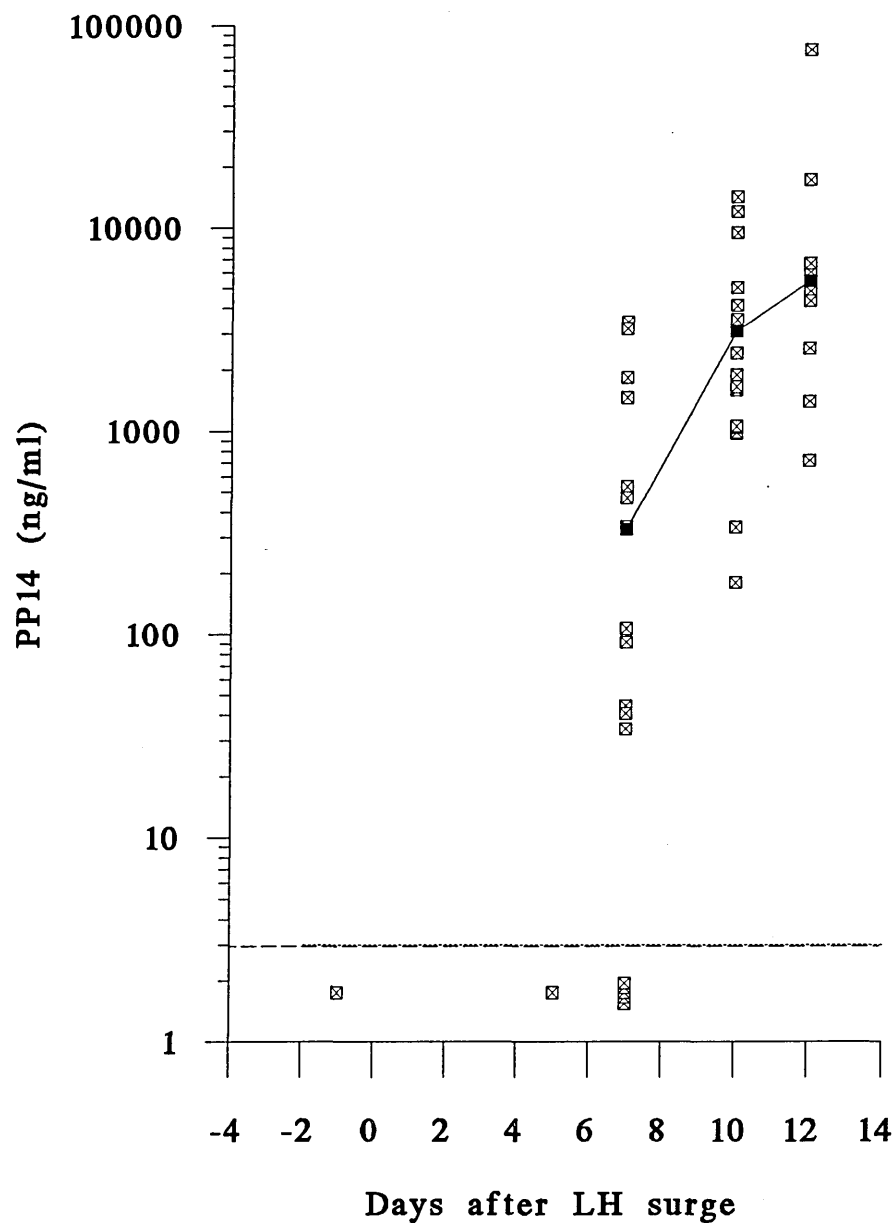
### 6.3.2 PP14 levels in uterine flushings and plasma samples from infertile women

A total of 49 uterine flushings and 48 plasma samples were taken from infertile women between days LH -1 and LH +12 of the menstrual cycle.

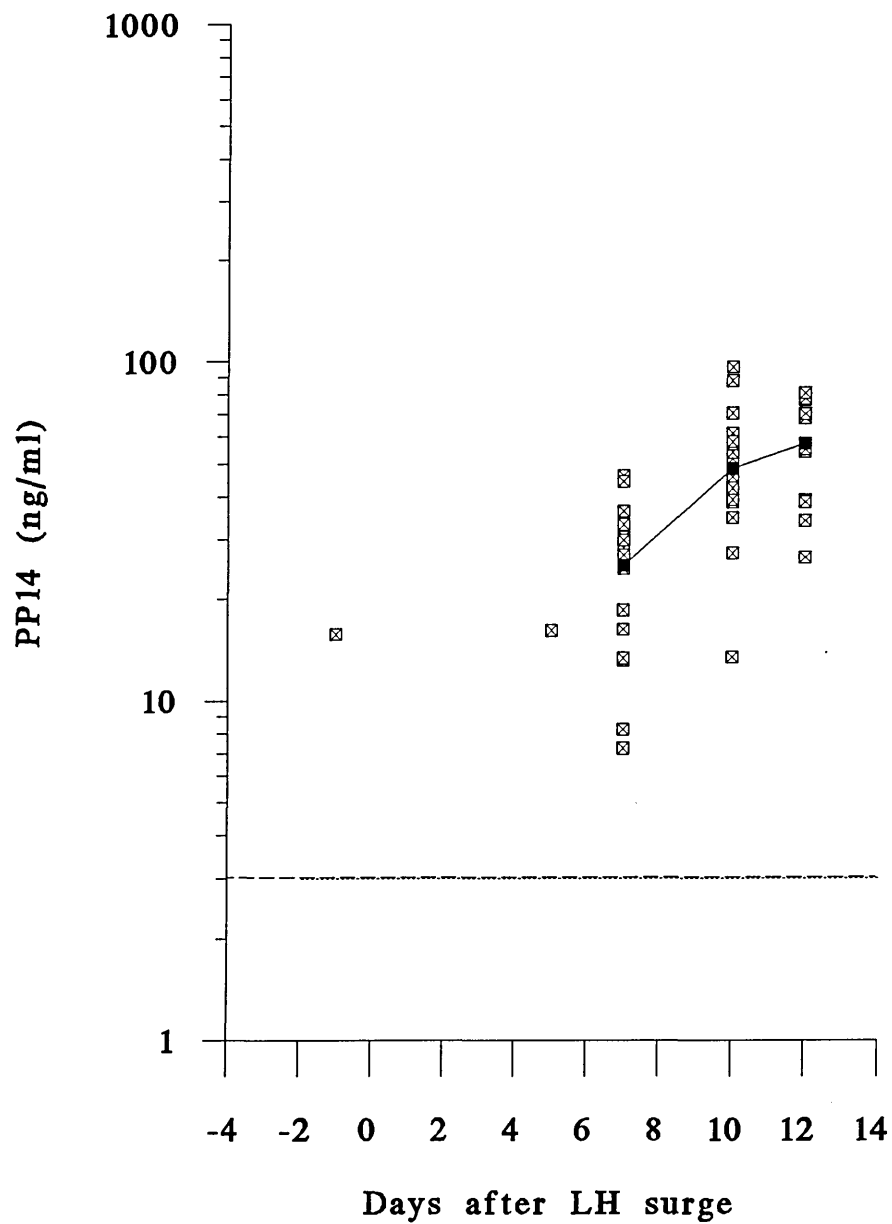
The concentrations of PP14 measured in uterine flushings from infertile women are shown in Figure 6.4. PP14 was undetectable in two samples taken before day LH +7. On day LH +7 PP14 was undetectable in 5 out of 17 samples (29%) compared to 1 out of 7 samples (13%) in fertile women. After day LH +7 the PP14 levels started to rise rapidly. The mean PP14 concentrations in uterine flushings were: LH +7 = 327.7 ng/ml, LH +10 = 3055.3 ng/ml, LH +12 = 5368.1 ng/ml.

The concentrations of PP14 measured in the plasma samples from infertile women are shown in Figure 6.5. As with the samples from fertile women PP14 was detectable in all plasma samples and the levels rose during the secretory phase of the menstrual cycle. The mean concentrations in the plasma samples were: LH +7 = 25.1 ng/ml, LH +10 = 48.2 ng/ml, LH +12 = 57.0 ng/ml.

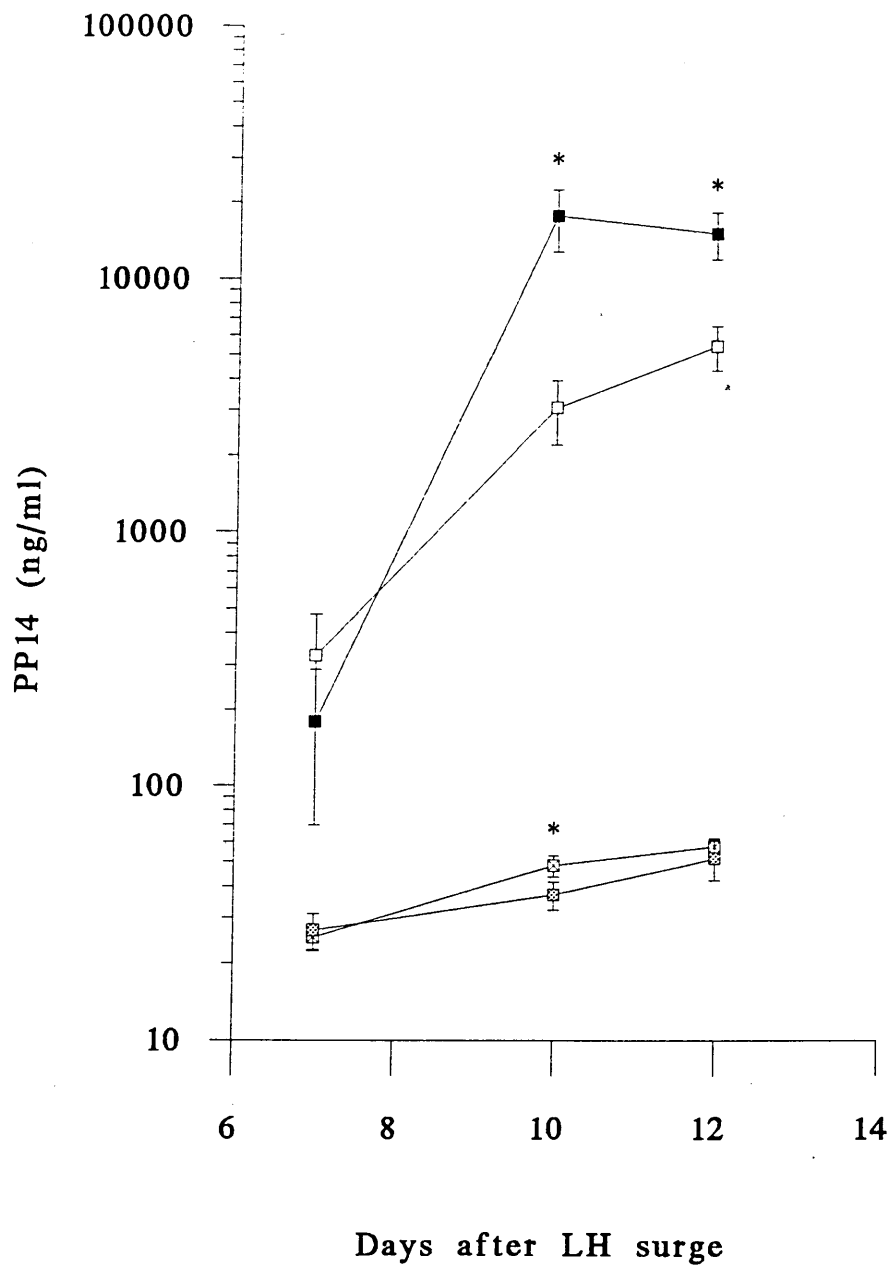
Figure 6.6 shows the comparison between the levels of PP14 measured in uterine flushings and in plasma samples from fertile and infertile women. The mean levels of PP14 in uterine flushings from infertile women were significantly lower than the levels measured in fertile women on day LH +10 ( $p > 0.005$ ) and day LH +12 ( $p > 0.005$ ). There was no significant difference seen between the levels measured on day LH +7. There was also no significant difference observed between the levels measured in the plasma samples on days LH +7 and LH +12, however on day LH +10 the plasma levels of PP14 from infertile women were significantly higher than those measured in fertile women ( $P < 0.05$ ).



**Figure 6.4:** The concentration of PP14 in uterine washings (n=49) obtained from 18 infertile women with regular menstrual cycles. On days where there were four or more results the means are represented by the closed squares. The dotted line indicates 3 ng/ml, the detection limit of the assay.



**Figure 6.5:** The concentration of PP14 measured in plasma samples (n=48) obtained from 18 infertile women with regular menstrual cycles. On days where there were four or more results the means are represented by the closed squares. The dotted line indicates 3 ng/ml, the detection limit of the assay.



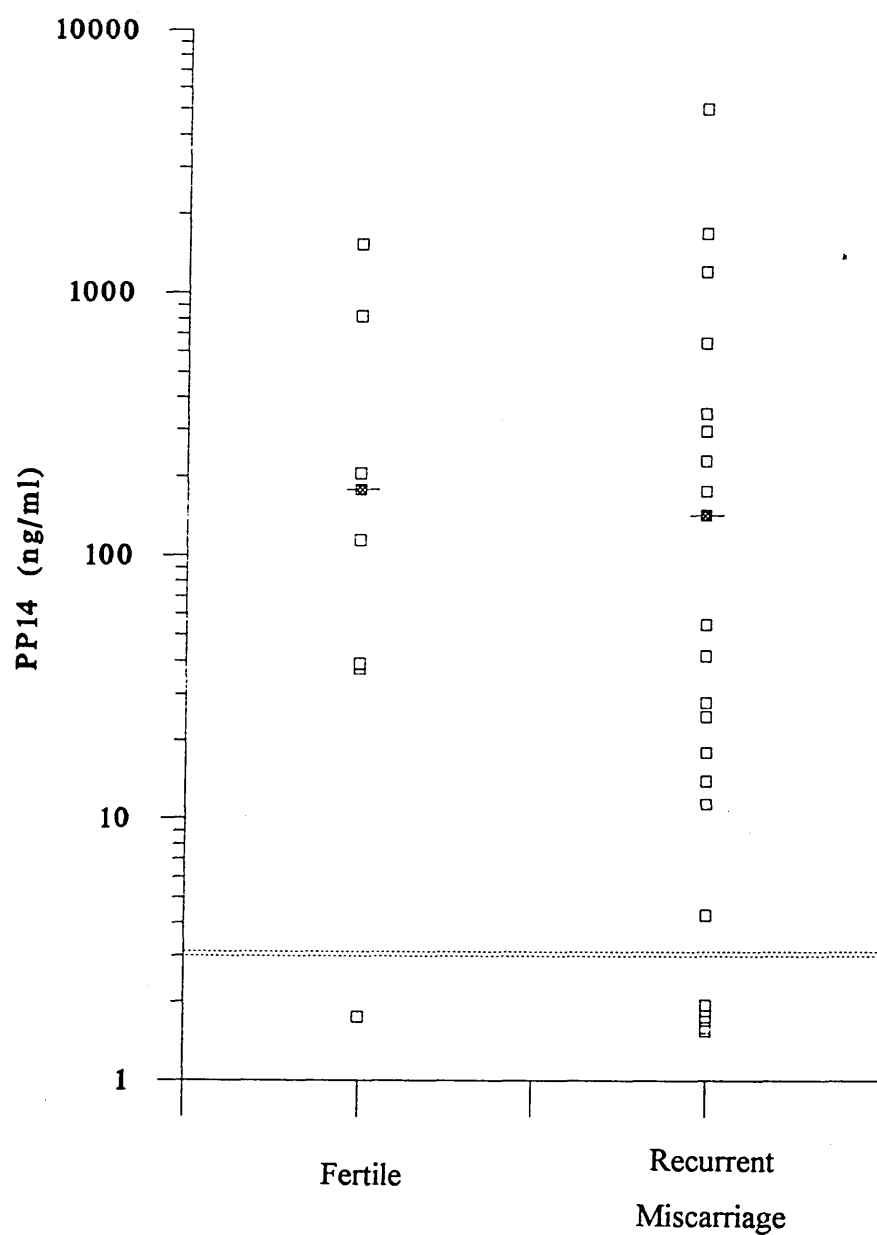
**Figure 6.6:** Comparison of the average levels of PP14 measured in uterine flushings (top graphs) and plasma samples (bottom graphs) from fertile and infertile women. The closed squares indicate the samples from fertile women, the open squares the samples from infertile women. The error bars represent the standard error of the mean for each set of samples. \* indicates statistical significance.

### 6.3.3 PP14 levels in uterine washings from recurrent miscarriage patients

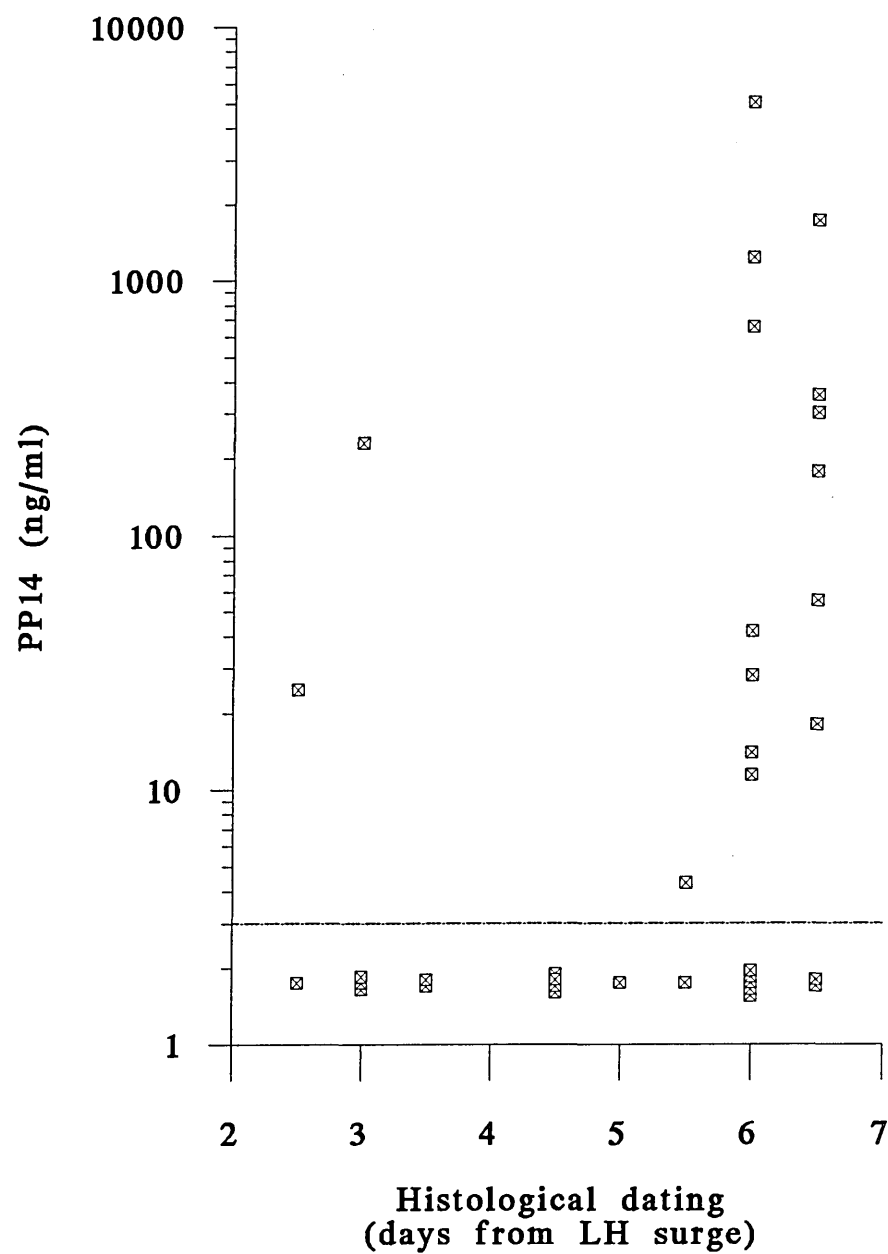
Figure 6.7 shows the levels of PP14 measured in uterine flushings from recurrent miscarriage patients on day LH +7 of the menstrual cycle. The levels of PP14 measured in uterine flushings from fertile women are also shown for comparison. There was no significant difference between the levels measured in uterine flushings from fertile women and recurrent miscarriage patients, however the range of values measured in the flushings from recurrent miscarriage patients was wider than that of the fertile women (<3-5000ng/ml, recurrent miscarriage, <3,-1540.7ng/ml, fertile women,) and the number of women with undetectable PP14 was higher in the recurrent miscarriage group (n=19, 54%) compared to the fertile group (n=1, 13%).

The PP14 levels measured in uterine flushings from women with primary and secondary miscarriage were compared. The data is summarised in table 6.1. The proportion of samples containing detectable PP14 was similar for both groups (primary, 44%; secondary, 50%) as were the means (primary, 58.4ng/ml; secondary, 65.1ng/ml).





**Figure 6.7:** Comparison of levels of PP14 measured in uterine flushings from 7 fertile women and 19 recurrent miscarriage patients. The dotted line indicates the detection limit of the assay 3ng/ml. The mean values are indicated by the lines. There was no significant difference between the levels for each group of women.



**Figure 6.8:** The relationship between PP14 concentrations in uterine flushings from recurrent miscarriage patients and the dating of endometrial biopsies taken on the same occasion.

	<b>PP14 Detectable (number of patients)</b>	<b>PP14 Undetectable (number of patients)</b>
<b>Primary miscarriage</b>	<b>11</b>	<b>14</b>
<b>Secondary miscarriage</b>	<b>5</b>	<b>5</b>

**Table 6.1:** Comparison of the detection and levels of PP14 in uterine flushings from primary and secondary miscarriage patients. Average PP14 levels: Primary miscarriage = 58.4ng/ml, S.E.M. = 31.2; Secondary miscarriage = 65.1ng/ml, S.E.M.= 40.3. Detection limit of the assay was 3ng/ml.

#### **6.3.4 Relationship between endometrial biopsy analysis and PP14 levels in uterine washings in recurrent miscarriage patients**

Figure 6.8 shows the levels of PP14 measured in uterine flushings taken from recurrent miscarriage patients immediately before an endometrial biopsy was taken, and the LH dating of that biopsy as assessed histologically by the method of Noyes (1950) or by morphometric analysis (Li *et al.*, 1988). PP14 was detectable in only 2 (17%) of the samples taken from women with retarded endometrium (n=12) but in women with normal endometrium PP14 was detected in 15 samples (65%, n=23). These data are summarised in Table 6.2. In the samples with detectable PP14 the mean levels were higher in the samples from women with normal endometrium than in the samples from those with retarded endometrium, however the difference between the two groups did not achieve significance.

#### **6.3.5 Subsequent pregnancy outcome of recurrent miscarriage patients related to PP14 levels**

Data were collected on the pregnancy outcome of the patients included in this study and related back to the levels of PP14 measured in uterine flushings taken during a previous menstrual cycle. 19 of the women subsequently became pregnant (54%). Of these women 14 had successful pregnancy leading to a live birth (74%) and 5 miscarried (26%). The levels of PP14 measured in the uterine flushings in these women are summarised in table 6.3. 4 of the 12 women with no detectable PP14 miscarried (33%) compared with 1 of the 7 women with detectable PP14 (14%). In the samples with detectable PP14 the mean levels of PP14 measured in the women who had a live birth were higher than the levels in the woman who miscarried but the numbers of patients are too small for this to be significant.

<b>Histological dating</b>	<b>PP14 Detectable (number of patients)</b>	<b>PP14 Undetectable (number of patients)</b>
<b>Normal (<math>\geq</math>LH+5)</b>	<b>14</b>	<b>9</b>
<b>Retarded (<math>&lt;</math>LH+5)</b>	<b>2</b>	<b>10</b>

**Table 6.2:** Detection of PP14 in uterine flushings from recurrent miscarriage patients. Mean PP14 levels: Normal endometrium = 683.2ng/ml; S.E.M. = 359.4; Retarded endometrium = 127.1ng/ml; S.E.M. = 102.4. Detection limit of the assay was 3ng/ml.

	PP14 Detectable	PP14 Undetectable
Live Birth	6	8
Miscarriage	1	4

**Table 6.3:** Detection of PP14 in uterine flushings and subsequent pregnancy outcome. Mean PP14 levels: Live birth = 1179.1ng/ml, S.E.M = 961.9; Miscarriage = 229.4ng/ml. Detection limit of the assay was 3ng/ml.

## 6.4 DISCUSSION

The measurement of PP14 in plasma samples has been shown to have limited use as a method of assessing endometrial function (*McRae et al., 1991*). This study investigated the use of the technique of uterine flushing coupled with the measurement of PP14 as a potentially useful way of investigating the local secretory activity of the endometrium.

### 6.4.1 PP14 levels in plasma samples and uterine flushings from normal, fertile women

PP14 levels were measured in plasma and uterine flushings from 23 proven fertile women. The results from the plasma samples confirmed previous studies (*Joshi et al., 1980; Julkunen et al., 1986b*), showing a general trend of rising plasma PP14 concentrations during the secretory phase of the menstrual cycle, with average levels doubling between days LH +7 and LH +12.

The PP14 levels observed in the uterine flushings showed a more pronounced pattern. In the two samples taken on day LH -3 PP14 was detectable, then the average levels dropped with the majority of samples between days LH -1 and LH +5 containing no detectable PP14. At day LH +7 onwards the levels of PP14 began to rise exponentially, with an mean doubling time of 13.7 hours between days LH +7 and LH +12. By day LH +10 the levels of PP14 in the uterine flushings were in the  $\mu\text{g/ml}$  range. The volume of fluid in the uterine cavity has been estimated as no more than 0.2ml (*Casslen, 1986*) therefore flushing the cavity with 10ml of fluid as was the case in this study would dilute this fluid by a factor of 50. Therefore the PP14 levels actually in the undiluted uterine fluid can be estimated to average  $875\mu\text{g/ml}$  at day LH+10. This is over 1000x higher than the mean PP14 levels measured in plasma samples at this time in the cycle. It may also be significant that the concentrations reached from day LH +7 onwards during the peri-implantation period are in the range at which PP14 is biologically active as shown in chapter 3. This points to a possible role for PP14 in the process of implantation.

There is a significant correlation between the levels of PP14 measured in plasma samples and that measured in uterine flushings taken on the same occasion in fertile women. Although it has been suggested that the endometrium may not be the only source of PP14 (*Seppälä et al., 1987*) the increase in plasma PP14 observed during the secretory phase of the menstrual cycle is likely to be due to seepage of PP14 from the uterine cavity into the plasma.

#### **6.4.2 PP14 levels in plasma samples and uterine flushings from infertile women**

PP14 levels were measured in plasma samples and uterine flushings from infertile women and compared to those observed in fertile women. There was no significant difference in the mean plasma levels between the two groups of women on days LH +7 and LH +12 but on day LH +10 the mean levels in the samples from infertile women were just significantly higher than the mean levels in the fertile women. In the uterine flushings on day LH +7 there was no significant difference in mean PP14 levels between the two groups while on days LH +10 and +12 the mean levels of PP14 in the uterine flushings from infertile women were significantly lower than those measured in flushings from fertile women.

Abnormalities in the secretory ability of the endometrium have been implicated as a cause of unexplained infertility (*Li et al., 1990*). The lower levels of PP14 in the uterine flushings from infertile women as compared to the levels of PP14 in the flushings from fertile women may be a reflection of this. The plasma levels did not show up this difference, confirming that plasma levels of PP14 are not sensitive enough to reflect changes in the local endometrial environment.

#### **6.4.3 PP14 levels in uterine flushings from recurrent miscarriage patients**

The levels of PP14 measured in uterine flushings from recurrent miscarriage patients was compared to the levels measured in flushings from fertile women on day LH +7, the day of implantation. There was no significant difference between the mean levels from the two groups, however the range of PP14 levels measured was wider in the samples from



women with recurrent miscarriage. This indicates that the secretory function of the endometrium in these patients may be out of phase, either ahead or behind the LH dating, leading to both higher and lower PP14 levels than those observed in fertile women.

The data was also analysed to see if there was a difference in the PP14 levels measured in uterine flushings from primary and secondary miscarriage patients. There was no difference observed between these groups. PP14 was equally likely to be detected and there was no significant difference in the mean PP14 levels measured or the S.E.M between the two groups.

The relationship between the levels of PP14 measured in the uterine flushings and the histological dating of biopsy samples taken immediately afterwards was investigated. There was not a clear correlation between the PP14 levels and the histological dating, instead there appear to be three groups of patients, all but two patients fit into one of three categories. The first group have normal endometrial development and also secrete measurable quantities of PP14. The second group have retarded endometrial development and PP14 cannot be detected in their uterine flushings. In both these groups the PP14 levels reflect the histological endometrial development. In the third group of patients this is not the case. These patients have apparently normal endometrial development as assessed histologically, but they do not have detectable levels of PP14 in their uterine flushings.

This implies that PP14 is a marker of endometrial secretory function, rather than endometrial development alone.

The pregnancy outcome of the recurrent miscarriage patients was compared with the levels of PP14 in the uterine flushings to see if PP14 had any predictive value. The numbers of patients were too low to draw any firm conclusions. However, it appears that although a lack of PP14 does not make a patient more likely to miscarry, the

detection of PP14 in uterine washings raises the probability that the pregnancy will be successful. 80% of patients who had detectable PP14 in their uterine flushings went on to have a successful pregnancy outcome compared to 66% of those without detectable PP14. This is likely to be due to PP14 secretion being a marker of endometrial development and secretory ability, although it may be a more direct effect.

When the study was initially designed LH +7 was picked as the day to study because it is around the time of implantation. The results from the fertile women show that PP14 secretion is only just detectable on this day and differences between these levels and those in infertile women only become significant later in the secretory phase. This may also be true for recurrent miscarriage patients and studies are currently underway to extend this work in this way.

## 6.5 CONCLUSION

From these results it would seem that PP14 measurement in uterine flushings is a more effective assessment of endometrial function than plasma PP14 measurement. In infertile patients PP14 measurements may be able to identify women with secretory dysfunction. This may also be true for women with recurrent miscarriage, and a lack of PP14 may indicate either endometrial retardation or secretory dysfunction. Further studies into PP14 and other secretory molecules in uterine flushings may distinguish between histological and secretory endometrial development and provide a non-invasive way of investigating the local uterine environment from a structural and a functional perspective.

# **CHAPTER SEVEN: FINAL DISCUSSION AND CONCLUSIONS**

## **7.1 INTRODUCTION**

### **7.1.1 Leukocytes in the decidua**

In Chapter 1 the presence in the decidua of NK-like large granular lymphocytes (LGLs) and macrophages was described. Both these cell populations appear to be specifically recruited, and are likely to have a role in the process of normal pregnancy. One function that has been suggested for the LGLs is that of surveillance of aberrant trophoblast, thereby controlling the invasion of the fetal tissue. These LGLs appear to be activated, which may be important for their function, but there does not appear to be a upregulation of the responses usually associated with immunologically activated cells. Therefore there appears to be a tightly regulated system that allows these activated cells to exist alongside the semi-allogenic fetus without a rejection response occurring. However if decidual LGLs are exposed to exogenous interleukin-2 they are then able to lyse trophoblast cells. They also release  $\gamma$ -interferon ( $\gamma$ -IFN), a cytokine which enhances the expression of MHC antigens, and may increase the susceptibility of the fetal tissue to this recognition process.

Activated lymphocytes release interleukin-2, which further stimulates the proliferation of lymphocytes in a positive feedback mechanism. The presence in the decidua of activated LGLs that do not appear to secrete IL-2 is intriguing. One explanation is the proposal that immunomodulators in the decidua suppress the immune response, preventing the upregulation of the immune response.

### **7.1.2 Immunomodulation**

While the observation that decidual tissue contains immunosuppressive activity is generally accepted, there is disagreement and controversy about which molecules are

responsible. Transforming growth factor- $\beta$ , an immunosuppressive cytokine, has been suggested as one of the molecules involved. More controversial is the suggested role of placental protein 14 (PP14), to which immunosuppressive activity has also been attributed (*Bolton et al., 1987*). PP14 is a major secretory product of the endometrium during the second half of the menstrual cycle and the decidua during the first trimester of pregnancy.

The study of immunomodulators from decidual tissue including PP14 may have important clinical applications, not only in the field of reproduction, but also in the study or treatment of diseases which involve upregulated inflammatory responses, such as rheumatoid arthritis.

### **7.1.3 Placental protein 14**

Most previous studies into PP14 have involved either the measurement of the molecule in plasma or serum in a clinical context, or investigations into the immunosuppressive activity of the molecule.

The aim of this thesis was to broaden the knowledge of PP14 and develop a number of ways of studying this molecule that would provide tools for future investigations. Studies were carried out to extend previous investigations of the activity, structure and clinical relevance of PP14. In addition a number of new methods of studying PP14 were developed. These included the U937 bioassay, the expression of PP14 as a recombinant protein and the technique of uterine flushing as a method to study PP14 in a clinical context.

## 7.2 PURIFICATION OF PP14 AND INVESTIGATION OF ACTIVITY

The purification method described in Chapter 2 yielded active PP14 at a purity of 97% in mg quantities. One key factor in the success of this method in recovering active protein may have been the maintenance of all stages of the method at 4°C. This could explain why some other investigators have failed to prepare active PP14 using methods which were carried out at room temperature.

The purified protein was investigated for its ability to suppress the response of peripheral blood mononuclear cells to IL-2. This is relevant because of the principal role of IL-2 in the immunological environment of the endometrium. The fact that PP14 blocks the proliferative response of PBMCs to IL-2 confirms its potential role in the immunomodulation of this environment.

PP14 may mediate its action by acting on monocyte-macrophages to suppress the release of IL-1. The suppression of the growth of the IL-1 dependent cell line U937 by PP14 would seem to confirm this theory.

However, the main point of the investigation into the suppression of U937 growth by PP14 was the potential this observation provided for the development of a bioassay for PP14. The lack of reproducibility was a serious disadvantage of the previous methods of investigating the activity of PP14. This was a natural consequence of the variation from one person to another of the response of blood cells to mitogens or PHA. The U937 assay provides a rapid, simple method for the further investigation of PP14. This assay can be used for the study of the stability of PP14, as demonstrated in Chapter 3. Other applications are the study of interaction of PP14 with receptors and the binding of PP14 to a co-factor or ligand.

The assay also enables the activity of PP14 to be defined in terms of Unit/ml. An arbitrary definition was made that 1 Unit of activity is the amount of PP14 activity

required to suppress by 50% the growth of  $1 \times 10^5$  U937 cells. The U937 assay described is not specific for PP14, but it can be made specific by the use of the immunoabsorbent gel.

### 7.3 EXPRESSION OF RECOMBINANT PP14 AND MOLECULAR MODELLING

The expression of a protein in a recombinant form is an important development towards the use of that protein as a therapeutic molecule. Although pure recombinant PP14 was not obtained, the cloning and expression of the cDNA coding for PP14 in *E. coli* provides the basis for a source of recombinant protein in the future. The optimisation of the expression and growth conditions has been achieved. Once the optimisation of the purification of the fusion protein, and the cleavage reaction have also been carried out a source of recombinant PP14 will be available.

An important factor in the activity of the recombinant preparation will be the requirement or otherwise of a co-factor or ligand for activity.

With this in mind, the molecular modelling of PP14 has been carried out using a computerised modelling programme. This enabled the investigation of the structure of PP14 and the binding pocket of the molecule. It also provided an opportunity to anticipate which co-factors or ligands may fit into the binding pocket.

Progesterone emerged as a potential ligand for PP14, fitting the binding pocket and having charged groups at either end which could interact with the charged amino acids that are present in those specific areas of the molecule.

In addition the modelled structure of PP14 predicted that the molecule shares many structural features with other members of the lipocalin family. As the mode of action of these other members is discovered this may assist with investigations into the mode of action of PP14. The model showed two possible receptor binding sites, one at the back of the binding pocket, the other at the entrance of the binding pocket.



## 7.4 CLINICAL STUDIES

The clinical data presented here demonstrates the importance of PP14 measurements in uterine flushings as a technique for investigating the local environment of the endometrium.

The levels of PP14 measured in uterine flushings from fertile women showed a pattern of secretion consistent with the previously reported immunohistochemical data. This pattern of secretion is observed in plasma PP14 measurements but the levels in uterine flushings are more directly representative of the secretory activity of the endometrium.

Infertile women had lower levels of PP14 and women suffering from recurrent miscarriage often did not secrete PP14 at all on day LH +7. The measurement of PP14 in these patients may diagnose endometrial secretory dysfunction.

## 7.5 EXTENSION OF THE KNOWLEDGE OF PP14

This thesis has described a number of investigations into the immunomodulatory protein PP14.

The overall understanding of this molecule was advanced in several key areas:

1. PP14 suppresses the proliferative response of peripheral blood mononuclear cells to IL-2. This is directly relevant to the situation in decidual tissue where an activation of lymphocytes by IL-2 may cause trophoblast lysis and fetal demise.
2. The suppression of growth of the cell line U937 can be used as the basis for a bioassay for PP14. This assay is reproducible, rapid and simple. It provides a method of expressing the specific activity of PP14.
3. PP14 has been expressed in a recombinant form, this is vital for any therapeutic application of the molecule. The availability of recombinant PP14 will also assist in the study of the activity of the molecule by providing an alternative source of pure protein.
4. The predicted molecular model of PP14 reveals that it is similar in structure to the other members of the lipocalin family. This identifies it as a carrier of small hydrophobic molecules. This characteristic could be exploited and PP14 used to carry a variety of molecules by modifying the amino acids inside the binding pocket.
5. PP14 measurement in uterine flushings provides a more accurate reflection of the secretory activity of the endometrium than plasma PP14 measurement. Reduced levels of PP14 are found in the flushings of infertile women, while in recurrent miscarriage patients PP14 is often absent in flushings in the peri-implantation period.

## 7.6 FUTURE STUDIES

As previously mentioned the investigations and results described in this thesis provide new methods by which to study PP14.

Some aspects of the studies need completion. A complete investigation of the stability of the activity of PP14, using the bioassay, would be one such study. The completion of the purification of recombinant PP14 is another.

The modelled molecular structure of PP14 can be used to investigate a wider and more extensive range of ligands. Solving the structure of PP14 by X-ray crystallography would be a useful confirmation of the data presented here.

An investigation into the levels of PP14 found in recurrent miscarriage patients later in the menstrual cycle would demonstrate whether these women, like those with unexplained infertility, secrete subnormal amounts of PP14. The technique of uterine flushing and subsequent PP14 measurement is more sensitive to changes in the uterine environment than the measurement of PP14 in plasma. This technique may be able to detect endometrial dysfunction, or the response of the endometrium to hormone therapies.

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## APPENDIX I REAGENTS, SUPPLIERS AND EQUIPMENT

### I.i Reagents

All reagents unless otherwise indicated were obtained from Sigma, Poole, Dorset, U.K.; BDH, Poole, Dorset, U.K.; or Fisons, Loughborough, Leicestershire, U.K.

DEAE Sepharose Fast Flow	Pharmacia Biochemicals, Hertfordshire, U.K.
Hexyl agarose	ACL chromatography, Isle of Man
Superdex 200 HR	Pharmacia Biochemicals, Hertfordshire, U.K.
Hiload Q Sepharose	Pharmacia Biochemicals, Hertfordshire, U.K.
Protein assay	Pierce, Chester U.K.
Molecular weight markers	Biorad, Hertfordshire, U.K.
Lymphopaque	Nycodenz, Sweden
AIM V medium	Gibco, Paisley U.K.
Scintillation fluid	Wallac, Milton Keynes, Hertfordshire, U.K.
mRNA extraction kit:	Stratagene, Cambridge, U.K.
Phenol	Appligene, Chester-le-Street, Co. Durham, U.K.
Chloroform	New Brunswick Scientific, Hatfield, U.K.
First-Strand cDNA synthesis kit	Pharmacia Biochemicals, Hertfordshire, U.K.
Restriction enzymes	Northumbria Biologicals Limited, Cranlington, Northumbria, U.K.
Taq Polymerase	Promega, Southampton, U.K.
T4 Ligase	Promega, Southampton, U.K.
GeneClean	Bio 101. California, U.S.A.
Agarose	BRL, Gaithersburg, Maryland, U.S.A.
Disposable plastics	Fisons, Leicester, U.K. Sarstedt Leicester, U.K. Alpha, Eastleigh, Hampshire, U.K. Costar, Buckinghamshire, U.K.

Nitrocellulose membranes (Hybond C)	Amersham, Little Chalfont, Buckinghamshire, U.K.
Filter papers	Whatman, Maidstone, Kent
Glutathione Agarose	Pharmacia Biochemicals, Hertfordshire, U.K.
Yeast extract	Oxoid, Unipath Ltd, Basingstoke U.K.
Tryptone	Difco, Detroit, Michagen, U.S.A.
Agar	Difco, Detroit, Michagen, U.S.A.
$^{125}\text{I}$ -NaOH	ICN Flow, Oxfordshire, U.K.
$^3\text{H}$ -Thymidine	ICN Flow, Oxfordshire, U.K.
Donkey anti-rabbit Amerlex	Amersham Little Chalfont, Buckinghamshire, U.K.

### **I.ii Equipment**

Gamma counter	LKB
Beta counter	LKB
Cell harvester	ICN Flow
Centrifuges	Centaur 2 (MSE) Beckman JA-21
Dri-Block	Techne
SDS-PAGE equipment	Biorad MiniProtean II
Power supplies	Pharmacia EPS 500/400 Hoefer, PS 500XT, (Hoeffer Scientific Instruments), San Francisco, U.S.A. Biorad Model 200/2.0.
Agarose gel electrophoresis	Biorad mini and miniwide sub DNA cell
Balances	Mettler AJ100
Western blot equipment	Hoefer TE Series Transphor
Vortex Mixer	MT20, Chiltern
Water Bath	Grant Cambridge
Rotary evaporator	Hetovac



Pipettes

Oligonucleotide synthesiser

Thermal cycler

Spectrophotometer

Eppendorf,

Applied Biosystems 392 DNA/RNA synthesiser

Coy Tempcycler, Flowgen

Ultraspec 4050 LKB

## **APPENDIX II PP14 RADIOIMMUNOASSAY**

### **II.i Iodination of PP14**

PP14 was measured using the method of Bolton et. al. (1983). PP14 was iodinated by the chloramine T method. 10µg of PP14 purified by the method described in chapter 2 in 10µl of iodination buffer (50mM phosphate buffer, pH 7.5) was placed in a 12mm x 75mm polystyrene tube. To this was added 1mCi of  $^{125}\text{I}$ -NaOH in 10µl, followed by 1.7 µg of chloramine T in 10µl of iodination buffer. After exactly 30 seconds the reaction was stopped by the addition of 5 mg of cysteine in 100µl of iodination buffer, followed by 10 mg NaI in 1ml of assay buffer (50mM phosphate, pH 7.5, containing 1% BSA and 0.1% sodium azide). The tracer was separated from the free iodine by applying it to a 10ml G25 column. The fractions containing protein were further purified on a 1ml column of concavalin A, to which PP14 binds. Damaged PP14 passes through and the bound PP14 was eluted with 0.2M mannose in assay buffer.

The binding of the tracer was titrated against the polyclonal antibody. The dilution of antibody which gave 50% binding of the tracer was picked to use in the assay.

### **II.ii PP14 radioimmunoassay**

100µl of each sample or standard (200-1.5ng/ml) was placed in a tube in duplicate. To this was added 100µl of PP14 tracer diluted to 1ng/ml in assay buffer and 100µl of polyclonal antibody diluted to give 50% binding of the tracer. Zero standard tubes containing assay buffer instead of sample, non-specific binding tubes containing assay buffer instead of sample and antibody and totals tubes containing tracer only were also set up. All tubes were mixed and left at room temperature overnight. Next 100µl of Amerlex antirabbit magnetic separating reagent was added to each tube except the totals tubes, the tubes were mixed and left to stand for 20 minutes. After centrifuging at 2000g for 10 minutes at room temperature the unbound fraction in the supernatant was aspirated and the bound fraction in the pellet counted in a gamma counter. The

detection limit of the assay was 3ng/ml and the inter- and intra-assay variation less than 10%.

## **APPENDIX III MONOCLONAL ANTIBODIES**

### **III.i Subcloning of monoclonal antibodies**

A cell line producing monoclonal antibodies against PP14 was subcloned. The cloning medium was RPMI supplemented with 20% FCS, 10% human endothelial cell supplement (HECS), 200mM Penicillin, 200µg/ml streptomycin and 2mM glutamine. Cells were diluted in this medium to concentrations of 320,160,80,40,20,10,5, and 2.5 cells /ml. 100µl of each dilution was placed in a row of wells in a 96 well plate. The wells were all topped up with an additional 100µl of cloning medium. A few hours later the wells were inspected microscopically and a note made of the number of cells present in each well. The plates were incubated for about a week until colonies of cells had grown. The cells were then subcultured into 24 well plates in RPMI + medium (RPMI, 10% fetal calf serum, 200mM penicillin, 200µg/ml streptomycin, 2mM glutamine).

After 3-4 days a portion of the supernatant was removed and tested for antibody activity against PP14. 100µl of iodinated PP14 diluted to 1ng/ml was mixed with 100µl of cell supernatant. After overnight incubation the bound tracer was separated from the unbound using Amerlex anti-mouse solid phase. Radioactivity was found in the bound fraction in clones secreting the antibody. Clones that tested positive were expanded into 25cm<sup>2</sup> flasks. 3-5 clones were selected, based on the extent of their binding to PP14, the fact that they originated from one cell only (as recorded when the wells were scored for cell numbers), and speed of growth. These clones were tested for subtype using a Serotec subclone kit and found to be subtype IgG1a. A portion of the expanded cells were frozen in liquid nitrogen in a freezing medium of 10% DMSO, 90% FCS. These clones were maintained as a stock cell line and expanded to produce monoclonal antibodies as required.

### **III.ii Production and purification of monoclonal antibodies**

The subcloned cells were expanded into 225cm<sup>2</sup> flasks in RPMI + medium. The cells were grown on to exhaustion, removed by centrifugation and up to 5 litres of supernatant mixed with an equal volume of saturated ammonium sulphate at 4°C. After 1 hour the suspension was centrifuged at 10,000g for 30 minutes at 4°C. The pellet containing the antibodies was resuspended in approximately 200mls of water and dialysed overnight against two changes each of 4 litres of PBS.

After centrifuging to remove any insoluble material the dialysed solution was applied to a 5ml Protein G column. After washing the column with 2 column volumes of PBS the antibodies were eluted with glycine-HCl (0.1M, pH 2.7). The eluted antibodies were collected as 1 ml fractions in tubes containing 100µl of 2M Tris pH 9.0 to protect against denaturation due to the low pH. The antibody concentration was measured using a RID kit and the antibodies were stored at -20°C until required.

## **APPENDIX IV SDS-PAGE ELECTROPHORESIS AND WESTERN BLOTTING**

### **IV.i SDS-PAGE**

SDS-PAGE was carried out using the Biorad mini-protean system. 12% gels were prepared by mixing the following reagents to make the separating gel:

8.1 ml acrylamide/bisacrylamide solution (30%, 29:1 w/w)

1.9 ml water

5.0 ml separating gel buffer (1.5M Tris-HCl, pH 8.8)

5 ml Ammonium persulphate (0.1%)

25µl TEMED

This mixture was poured in to the gel mould and overlayed with water saturated butanol to speed up the polymerisation which is inhibited by air. After it had set (approximately 1 hour) a stacking gel was poured consisting of the following reagents:

3.2ml acrylamide/bisacrylamide solution (30%, 29:1 w/w)

6.8ml water

5.0ml stacking gel buffer (0.5M Tris-HCl, pH 6.8)

5 ml Ammonium persulphate

25µl TEMED

A comb to form the well was inserted into the stacking gel immediately after it had been poured and the gel was left to set for 1 hour.

After loading the samples the gels were electrophoresed for 1 hour at 150V then stained in 0.25% Coomassie Brilliant Blue stain for 1 hour. The gels were destained in methanol:water:acetic acid (5:5:1).

#### **IV.ii Western blotting**

After running the gels some lanes were blotted. The lanes were sliced from the rest of the gel which was then stained. Nitrocellulose membrane was cut to the same size as the gel and placed on top of it. The gel and membrane were sandwiched between four pieces of filter paper soaked in separating buffer, placed in a semi-dry blotting apparatus and a current of 10-15mA/cm<sup>2</sup> of gel was applied for 20 minutes.

The nitrocellulose membrane was removed and the free binding sites were blocked by incubation in blotto (PBS, 2.5% skimmed milk powder, 0.1% sodium azide) overnight with shaking on a plate shaker at room temperature. The membrane was then incubated for 2 hours in blotto containing the primary antibody. After three ten minute washes in blotto with shaking the membrane was placed in blotto containing the secondary antibody conjugate (anti-rabbit alkaline phosphatase or anti-mouse alkaline phosphatase 1/10000 dilution). The membrane was incubated for two hours with shaking, then washed twice for 10 minutes with blotto then washed twice in substrate buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 50mM MgCl<sub>2</sub>). The substrate (BCIP/NBT, 1.5mg/3mg) was added to the membrane in substrate buffer and incubated for 30 minutes or until colour development was observed.

## **APPENDIX V PROTEIN ASSAY**

Total soluble protein was measured using a Pierce BCA protein kit adapted for use in microtitre plates.

10µl of samples or standards in duplicate were placed in the wells of a microtitre plate. 200µl of assay reagent (bicinchoninic acid and copper sulphate) was added to all wells. After shaking the plate on a plate shaker for 30 seconds the plate was covered and incubated at 37°C for 30 minutes. The colour development was read at 540nm in a plate reader. The standard protein used was albumin at concentrations in the range 200µg/ml-2000µg/ml.